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(21) International Application Number: PCT/US99/18539 (22) International Filing Date: 13 August 1999 (13.08.99) (30) Priority Data: 60/096,515 14 August 1998 (14.08.98) US (63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 60/096,515 (CON) Filed on 14 August 1998 (14.08.98) (71) Applicant (for all designated States except US): PRESIDENT AND FELLOWS OF HARVARD COLLEGE [US/US]; 17 Quincy Street, Cambridge, MA 02138 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): LEE, Mu-En [CN/US]; 102 Nardell Road, Newton, MA 02159 (US). MAEMURA, Koji [JP/US]; Apartment #638, 185 Freeman Street, Brookline, MA 02446 (US). HIESH, Chung-Ming [CN/US]; 1716 Cambridge Street, Cambridge, MA 02138 (US). (74) Agent: BEATTIE, Ingrid, A.; Fish & Richardson P.C., 225 Franklin Street, Boston, MA 02110-2804 (US).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: METHODS OF MODULATING OF ANGIOGENESIS (57) Abstract A method of inhibiting angiogenesis in a mammal by administering to the mammal a compound which inhibits binding of endothelial PAS domain protein-1 to cis-acting transcription regulatory sequence in the promoter region of a gene encoding an angiogenic factor.		

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METHODS OF MODULATING OF ANGIOGENESISStatement as to Federally Sponsored Research

5 This invention was made in part with funds from the Federal Government under National Institutes of Health grants HL 09008, HL 55454, HL 03194, HL 57664, GM 53249 and HL 57977.

Background of the Invention

10 This invention relates to vascular therapy.

 Angiogenesis results from endothelial cell proliferation induced by angiogenic factors. Angiogenic factors bind to receptors on endothelial cells which line blood vessels. This event triggers signals which cause
15 the cells to proliferate; the proliferating endothelial cells secrete proteases which digest the basement membrane surrounding a vessel. The junctions between the endothelial cells are altered, allowing projections from the cells to pass through the space created. These
20 outgrowths then become new blood vessels, e.g., capillaries.

 Vascular endothelial cell growth factor (VEGF) and VEGF receptors (VEGF-Rs) play a role in vasculogenesis and angiogenesis. Although VEGF is secreted by a variety
25 of cell types, including vascular smooth muscle cells, osteoblasts, fibroblasts, and macrophages, its proliferative and chemotactic activities are restricted to endothelial cells. VEGF signaling is mediated by two VEGF-Rs, the endothelial cell-specific tyrosine kinase
30 receptors, flt-1 and KDR/flk-1. Despite its importance in VEGF signaling, the molecular mechanisms of VEGF and VEGF-R expression have not been elucidated.

Summary of the Invention

 The invention is based on the discovery that
35 endothelial PAS domain protein-1 (EPAS1) binds to cis-acting regulatory sequences associated with genes

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encoding such angiogenic factors as VEGF and VEGF-Rs such as KDR/flk-1 and flt-1, thereby transactivating the promoters of such genes. Accordingly, the invention features a method of increasing the level of EPAS1 in a cell, e.g., an endothelial cell. An increase in the level of EPAS1 leads to increased promoter transactivation and increased transcription of genes encoding angiogenic factors which participate in the blood vessel formation.

10 The invention also includes a novel basic helix-loop-helix/Per-AhR-Arnt-Sim (bHLH/PAS) protein which binds to EPAS1 and forms a heterodimer which transactivates transcription of genes encoding angiogenic factors. Increasing the level of ARNT4 in a cell, e.g.,
15 an endothelial cell also leads to increased promoter transactivation and increased expression of angiogenic factors which participate in the blood vessel formation.

Angiogenic factors are proteins or polypeptides and ligands thereof that participate in the process of new blood vessel formation. For example, angiogenic factors include VEGF, VEGF-Rs, and other signalling proteins such as intracellular tyrosine kinases which participate in the angiogenic process. Preferably, the angiogenic factors are expressed in endothelial cells,
20 e.g., VEGF, VEGF-Rs such as KDR/flk-1 or flt-1, and tyrosine kinases such as Tie2.

A method of inhibiting angiogenesis in a mammal is carried out by administering to the mammal a compound which inhibits binding of EPAS1 to cis-acting
30 transcription regulatory DNA associated with a gene encoding an angiogenic factor. Angiogenesis is also inhibited by administering a compound which inhibits binding of EPAS1 to ARNT4, i.e., a compound which inhibits the formation of a functional heterodimer that
35 can transactivate a promoter of gene encoding an

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angiogenic factor. The angiogenic factor is preferably VEGF, a VEGF-R such as KDR/flk-1 or flt-1. For example, the compound inhibits transcription of the angiogenic factor by binding to a cis-acting regulatory sequence
 5 such as the sequence 5' GCCCTACGTGCTGTCTCA 3' (SEQ ID NO:1) in VEGF promoter DNA. For example, the compound is an EPAS1 polypeptide that binds to a cis-acting regulatory sequence of a gene but fails to transactivate the promoter of the gene, e.g, a polypeptide lacking a
 10 transactivation domain (amino acids 486-690 of EPAS1).

Table 1: Transactivation domain of human EPAS1

EDYYTSLDNDLKIEVIEKLFAMDTEAKDQCSTQTDNFELDLETLAPYIPMDGEDFQL
 SPICPEERLLAENPQSTPQHCFSAMTNIFQPLAPVAPHSPFLLDKFQQQLESKKTEP
 EHRPMSSIFFDAGSKASLPPCCGQASTPLSSMGGRSNTQWPPDPPLHFGPTKWAVGD
 15 QRTEFLGAAPLGPPVSPPHVSTFKTRSAKGFGAR (SEQ ID NO:2)

When such an EPAS1 mutant is bound to a cis-acting regulatory DNA, it prevents wild type EPAS1 binding and thereby inhibits transcription of a gene encoding an angiogenic factor (and, in turn, angiogenesis). For
 20 example, the EPAS1 polypeptide contains the N-terminal binding domain (amino acids 14-67 of EPAS1;
 RRKEKSRDAARCRRSKETEVFYELAHELPLPHSVSSHLDKASIMRLEISFLRTH;
 SEQ ID NO:3), more preferably the EPAS polypeptide contains amino acids 1-485 of human EPAS1. The amino
 25 acid sequence of such an EPAS1 dominant negative mutant polypeptide and DNA encoding such a mutant polypeptide is provided below.

Table 2: EPAS1 dominant negative mutant

1 MTADKEKKRS SSERRKEKSR DAARCRRSKE TEVFYELAHE
 30 LPLPHSVSSH
 51 LDKASIMRLE ISFLRTHKLL SSVCSENESE AEADQQMDNL
 YLKALEGFIA
 101 VVTQDGMIF LSENISKFMG LTQVELTGHS IFDFTHPCDH
 EEIRENLSLK

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151 NGSFGGKSK DMSTERDFM RMKCTVTNRG RTVNLKSATW
 KVLHCTGQVK
 201 VYNNCPPHNS LCGYKEPLLS CLIIMCEPIQ HPSHMDIPLD
 SKTFLSRHSM
 5 251 DMKFTYCDDR ITELIGYHPE ELLGRSAYEF YHALDSENMT
 KSHQNLCTKG
 301 QVVSGQYRML AKHGGYVWLE TQGTVIYNPR NLQPQCIMCV
 NYVLSEIEKN
 351 DVVFSMDQTE SLFKPHLMAM NSIFDSSGKG AVSEKSNFLF
 10 TKLKEEPEEL
 401 AQLAPTPGDA IISLDFGNQN FEESSAYGKA ILPPSQPWAT
 ELRSHSTQSE
 451 AGSLPAFTVP QAAAPGSTTP SATSSSSSCS TPNSP (SEQ ID NO:4)

Table 3: DNA encoding EPAS1 Dominant Negative Mutant

15 cctgactgcgcggggcgctcgggacctgcgcgcacctcggaccttcaccacccgccc
 gggccgcggggagcggacgagggccacagccccccaccgcccagggagcccaggtgc
 tcggcgtctgaacgtctcaaagggccacagcgacaatgacagctgacaaggagaaga
 aaaggagtagctcggagaggaggaaggagaagtcccgggatgctgcgcggtgccggc
 ggagcaaggaga
 20 cggaggtgttctatgagctggcccatgagctgcctctgccccacagtgtgagctccc
 atctggacaaggcctccatcatgcgactggaaatcagcttcctgcgaacacacaagc
 tcctctcctcagtttgctctgaaaacgagtcggaagccgaagctgaccagcagatgg
 acaacttgtaacctgaaagccttgagggtttcattgccgtggtgaccaagatggcg
 acatgatctttc
 25 tgtcagaaaacatcagcaagttcatgggacttacacaggtggagctaacaggacata
 gtatctttgacttcactcatccctgcgaccatgaggagattcgtgagaacctgagtc
 tcaaaaatggctctgggttttgggaaaaaaagcaaaagacatgtccacagagcgggact
 tcttcatgaggatgaagtgcacggtcaccaacagaggccgtactgtcaacctcaagt
 cagccacctgga
 30 aggtcttgactgcacggggcaggtgaaagtctacaacaactgcctcctcacaata
 gtctgtgtggctacaaggagcccctgctgtcctgcctcatcatcatgtgtgaaccaa
 tccagcaccatcccacatggacatccccctggatagcaagaccttcctgagccgcc

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acagcatggacatgaagttcacctactgtgatgacagaatcacagaactgattggtt
accaccctgagg
agctgcttggccgctcagcctatgaattctaccatgcgctagactccgagaacatga
ccaagagtaccagaacttgatgcaccaaggggtcaggtagtaagtggccagtaccgga
5 tgctcgcaaagcatgggggctacgtgtggctggagacccaggggacgggtcatctaca
accctcgcaacctgcagccccagtgcatcatgtgtgtcaactacgtcctgagtgaga
ttgagaagaatg
acgtgggtgttctccatggaccagactgaatccctgttcaagccccacctgatggcca
tgaacagcatctttgatagcagtggaagggggctgtgtctgagaagagtaacttcc
10 tattaccaagctaaaggaggagcccgaggagctggccagctggctcccacccag
gagacgccatcatctctctggatttcgggaatcagaacttcgaggagtcctcagcct
atggcaaggcca
tcctgcccccgagccagccatggggccacggagttgaggagccacagcaccagagcg
aggctgggagcctgcctgccttcaccgtgccccaggcagctgccccgggcagcacca
15 ccccgagtgccaccagcagcagcagcagctgctccacgcccgaatagcccttga
(SEQ ID NO:5)

Rather than administering EPAS1 polypeptides or ARNT4 polypeptides, the method may be carried out by administering DNA encoding such polypeptides. For
20 example, the compound is a nucleic acid encoding an EPAS1 polypeptide lacking amino acids 486-690 of EPAS1. For example, the nucleic acid encodes a dominant negative mutant of EPAS1 which contains amino acids 1-485 of wild type EPAS1, i.e., SEQ ID NO:5.

25 For antisense therapy, the compound is a antisense nucleic acid molecule containing at least 10 nucleotides the sequence of which is complementary to an mRNA encoding all or part of a wild type EPAS1 polypeptide. Preferably, the compound, e.g., an antisense
30 oligonucleotide or antisense RNA produced from an antisense template, inhibits EPAS1 expression. For example, the compound may inhibit EPAS1 expression by inhibiting translation of EPAS1 mRNA. For example, antisense therapy is carried out by administering a
35 single stranded nucleic acid complementary at least a

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portion of EPAS1 mRNA to interfere with the translation of mRNA into protein, thus reducing the amount of functional EPAS1 produced in the cell. A reduction in the amount of functional transactivating EPAS1 reduces the level of transcription of angiogenic factors such as VEGF or VEGF-Rs, resulting in a decrease in new blood vessel formation.

Alternatively, the compound is an EPAS1-specific intrabody, i.e., a recombinant single chain EPAS1-specific antibody that is expressed inside a target cell, e.g., a vascular endothelial cell. Such an intrabody binds to endogenous intracellular EPAS1 and prevents it from binding to its target cis-acting regulatory sequence in the promoter region of a gene encoding an angiogenic factor such as VEGF or a VEGF-R. An ARNT4-specific intrabody is also useful to inhibit angiogenesis.

Angiogenesis contributes to the progression of atherosclerotic lesions. Thus, compounds are administered to a site of an atherosclerotic lesion in a mammal to inhibit growth of a lesion. Compounds may also be locally administered to a tumor site to reduce blood vessel formation, thereby depriving a tumor of blood supply and inhibiting tumor growth. VEGF itself is a growth factor for some tumors; the methods described above directly inhibit VEGF expression, and thus, are particularly useful for treating such tumor types.

The invention also includes an antibody which binds to EPAS1. The antibody preferably binds to the C-terminal portion of EPAS1 (e.g., a polypeptide having the amino acid of SEQ ID NO:17 or 18). The antibody is a polyclonal or monoclonal antibody which specifically binds to the EPAS1. Preferably, the antibody binds to an epitope within the C-terminal transactivation domain (SEQ ID NO:2). The invention encompasses not only an intact monoclonal antibody, but also an immunologically-active

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antibody fragment, e.g., a Fab or (Fab)₂ fragment; an engineered single chain Fv molecule; or a chimeric molecule, e.g., an antibody which contains the binding specificity of one antibody, e.g., of murine origin, and
5 the remaining portions of another antibody, e.g., of human origin.

To promote angiogenesis in a mammal, a compound, e.g., DNA encoding EPAS1 or a functional fragment thereof, which increases expression of VEGF or a VEGF-R
10 in an endothelial cell is administered to a mammal, e.g., an adult mammal which has been identified as being in need of therapy to promote angiogenesis such as a patient suffering from peripheral vascular disease. A functional fragment of EPAS1 is one which binds to DNA in the
15 promoter region of a gene encoding an angiogenic factor.

The invention also features an EPAS-binding element, ARNT4 and a nucleic acid which encodes ARNT4. For example, the nucleic acid includes a sequence which encodes the amino acid sequence a naturally-occurring
20 human ARNT4 (SEQ ID NO:19). The DNA may encode a naturally-occurring mammalian ARNT4 polypeptide such as a human, rat, mouse, guinea pig, hamster, dog, cat, pig, cow, goat, sheep, horse, monkey, or ape ARNT4. Preferably, the DNA encodes a human ARNT4 polypeptide,
25 e.g., a polypeptide which contains part or all of the amino acid sequence of SEQ ID NO:19. The invention includes degenerate variants of the human cDNA (SEQ ID NO:20) . The DNA contains a nucleotide sequence having at least 50% sequence identity to SEQ ID NO:20. For
30 example, the DNA contains a sequence which encodes a human ARNT4 polypeptide, such as the coding sequence of SEQ ID NO:20 (nucleotides 220 to 2025 of SEQ ID NO:20). The DNA contains a strand which hybridizes at high stringency to a strand of DNA having the sequence of SEQ
35 ID NO:20, or the complement thereof. The DNA has at

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least 50% sequence identity to SEQ ID NO:20 and encodes a polypeptide having the biological activity of a ARNT4 polypeptide, e.g, the ability to bind to EPAS1 to form a heterodimer. Preferably, the DNA has at least 75% identity, more preferably 85% identity, more preferably 90% identity, more preferably 95% identity, more preferably 99% identity, and most preferably 100% identity to the coding sequence of SEQ ID NO:20.

Nucleotide and amino acid comparisons are carried out using the CLUSTAL W sequence alignment system with (Thompson et al., 1994, Nucleic Acids Research 22:4673-4680 or <http://www.infobiogen.fr/docs/ClustalW/clustalw.html>). Amino acid sequences were compared using CLUSTAL W with the PAM250 residue weight table. "Per cent sequence identity", as that term is used herein, is determined using the CLUSTAL W sequence alignment system referenced above, with the parameters described herein. In the case of polypeptide sequences which are less than 100% identical to a reference sequence, the non-identical positions are preferably, but not necessarily, conservative substitutions for the reference sequence. Conservative substitutions typically include substitutions within the following groups: glycine and alanine; valine, isoleucine, and leucine; aspartic acid and glutamic acid; asparagine and glutamine; serine and threonine; lysine and arginine; and phenylalanine and tyrosine.

Hybridization is carried out using standard techniques, such as those described in Ausubel et al. (*Current Protocols in Molecular Biology*, John Wiley & Sons, 1989). "High stringency" refers to nucleic acid hybridization and wash conditions characterized by high temperature and low salt concentration: wash conditions of 65°C at a salt concentration of 0.1 X SSC. "Low" to

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"moderate" stringency denotes DNA hybridization and wash conditions characterized by low temperature and high salt concentration: wash conditions of less than 60°C at a salt concentration of 1.0 X SSC. For example, high
5 stringency conditions include hybridization at 42°C, and 50% formamide; a first wash at 65°C, 2X SSC, and 1% SDS; followed by a second wash at 65°C and 0.1% x SSC. Lower stringency conditions suitable for detecting DNA sequences having about 50% sequence identity to an ARNT4
10 gene are detected by, for example, hybridization at 42°C in the absence of formamide; a first wash at 42°C, 6X SSC, and 1% SDS; and a second wash at 50°C, 6X SSC, and 1% SDS.

A vector containing an ARNT4-encoding DNA is also
15 within the invention. Preferably the DNA which includes an ARNT4-encoding DNA is less than 5 kilobases in length; more preferably, the DNA is less than 4 kilobases in length, more preferably the DNA is less than 3 kilobases in length, and most preferably the DNA is approximately 2
20 kilobases or less in length. The invention also provides a method of directing cardiac-specific or smooth muscle cell-specific expression of a protein by introducing into a cell an isolated DNA containing a sequence encoding the protein operably linked to the tissue-specific promoter.
25 A cell containing the DNA or vector of the invention is also within the invention.

By "substantially pure DNA" is meant DNA that has a naturally-occurring sequence or that is free of the genes which, in the naturally-occurring genome of the
30 organism from which the DNA of the invention is derived, flank the ARNT4 gene. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector, into an autonomously replicating plasmid or virus, or into the genomic DNA of a procaryote or
35 eucaryote at a site other than its natural site; or which

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exists as a separate molecule (e.g., a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. It also includes a recombinant DNA which is part of a
5 hybrid gene encoding additional polypeptide sequence.

Also within the invention is a substantially pure human ARNT4 polypeptide. The term ARNT4 polypeptide includes a polypeptide having the amino acid sequence and length of the naturally-occurring ARNT4 as well as
10 fragments of the full-length naturally-occurring ARNT4. The polypeptide contains the amino acid sequence of SEQ ID NO:19. Preferably the polypeptide contains an amino acid sequence which is at least 50% identical to SEQ ID NO:19. Preferably, the amino acid sequence has at least
15 75% identity, more preferably 85% identity, more preferably 90% identity, more preferably 95% identity, more preferably 99% identity, and most preferably 100% identity to the amino acid sequence of SEQ ID NO:19. For example, the ARNT4 polypeptide may have the amino acid
20 sequence of the naturally-occurring human polypeptide, e.g., a polypeptide which includes the amino acid sequence of SEQ ID NO:19. The invention also encompasses a polypeptide with the amino acid sequence of a segment of SEQ ID NO: 17 which spans residues 75 to 128,
25 inclusive, or a segment spanning residues 155 to 207, inclusive, of SEQ ID NO:19, or a segment spanning residues 232 to 384 of SEQ ID NO:19. Preferably, such a polypeptide has a biological activity of a naturally-occurring ARNT4 polypeptide, e.g, heterodimer formation
30 with EPAS1 or the ability to transactivate transcription under the control of a VEGF promoter.

A substantially pure ARNT4 polypeptide is obtained by extraction from a natural source; by expression of a recombinant nucleic acid encoding a ARNT4 polypeptide; or
35 by chemically synthesizing the protein. A polypeptide or

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protein is substantially pure when it is separated from those contaminants which accompany it in its natural state (proteins and other naturally-occurring organic molecules). Typically, the polypeptide is substantially
5 pure when it constitutes at least 60%, by weight, of the protein in the preparation. Preferably, the protein in the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, ARNT4. Purity is measured by any appropriate method, e.g.,
10 column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis. Accordingly, substantially pure polypeptides include recombinant polypeptides derived from a eucaryote but produced in *E. coli* or another procaryote, or in a eucaryote other than
15 that from which the polypeptide was originally derived.

The invention also includes a transgenic non-human mammal, the germ cells and somatic cells of which contain a null mutation in a gene encoding an ARNT4 polypeptide. For example, the null mutation is a deletion of part or
20 all of an exon of ARNT4. Preferably, the mammal is a rodent such as a mouse. An antibody which specifically binds to a ARNT4 polypeptide is also within the invention.

Angiogenesis is inhibited by administering to a
25 mammal a compound which inhibits binding of EPAS1 to ARNT4 such as an ARNT4 polypeptide. For example, the compound is a polypeptide or peptide mimetic which contains the amino acid sequence of residues 75 to 128, inclusive, of SEQ ID NO:19, the amino acid sequence of
30 residues 155 to 207, inclusive, of SEQ ID NO:19, or a the amino acid sequence of residues 232 to 384 of SEQ ID NO:19.

Other features and advantages of the invention will be apparent from the following detailed description
35 and from the claims.

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Detailed Description

The drawings will first be described.

Drawings

Fig. 1A is a bar graph showing dose-dependent
5 transactivation of KDR/flk-1 promoter by EPAS1. EPAS1
expression plasmid phEP-1 (0-6 μ g), pCDNA3 (6-0 μ g), and
KDR/flk-1 reporter pGL2-4kb+296 (1 μ g) were transfected
into BAEC.

Fig. 1B is a bar graph showing that deletion of
10 EPAS1 C-terminal region abolishes its ability to
transactivate the KDR/flk-1 promoter. Expression
plasmids (6 μ g each) and pGL2-4.0kb+296 (1 μ g) were
cotransfected into BAEC. For all constructs in Figs. 1A-
B, the plasmid pCMV- β GAL was cotransfected to correct for
15 differences in transfection efficiency. In both Figs. 1A
and 1B, luciferase activity and β -galactosidase activity
were measured, and normalized luciferase activity was
calculated as described below. The "fold induction"
represents the ratio (mean \pm SE) of normalized luciferase
20 activity in cells transfected with expression plasmid to
that in cells transfected with empty vector (pCDNA3).

Fig. 2A is a bar graph showing transactivation of
the KDR/flk-1 promoter by EPAS1 but not by HIF-1 α
(another member of the PAS family of transcription
25 factors). Expression plasmids (6 μ g each) and KDR/flk-1
reporter pGL2-4kb+296 (1 μ g) were cotransfected into the
cell types indicated.

Fig. 2B is a bar graph showing transactivation of
a VEGF promoter by EPAS1 and HIF-1 α . Expression plasmids
30 (6 μ g each) and VEGF reporter pVR47/CAT (1 μ g) were
cotransfected into the cell types indicated. For all
constructs in Figs. 2A-2B, the plasmid pCMV- β GAL was
cotransfected to correct for differences in transfection
efficiency. The "fold induction" represents the ratio
35 (mean \pm SE) of normalized luciferase or CAT activity in

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cells transfected with expression plasmid to that in cells transfected with empty vector (pcDNA3).

Fig. 3A is a diagram showing an alignment of the amino acid sequence of human ARNT4 with human BMAL1b and 5 human ARNT.

Fig. 3B is a diagram of a phylogenetic tree of the ARNT family of proteins.

Fig. 4 is a bar graph showing the results of a yeast two-hybrid assay. ARNT3 (BMAL1b) and ARNT4 form 10 heterodimers with EPAS1 as well as with CLOCK.

Fig. 5 is a bar graph showing that EPAS1 interacts with ARNT4 to form functional heterodimers which increase VEGF promoter activity and VEGF expression.

Fig. 6 is a bar graph showing that ARNT4 does not 15 interact with HIF-1 α .

Description of the Preferred Embodiments

EPAS1 is a member of the transcription factor family characterized by a basic helix-loop-helix (bHLH) domain and a (Per-AhR-Arnt-Sim) PAS domain composed of 20 two imperfect repeats. Table 4 shows the amino acid sequence of human wild type EPAS1.

Table 4: Amino acid sequence of human EPAS1

MTADKEKKRSSSERRKEKSRDAARCRRSKETEVFYELAH~~ELPLPHSVSSHL~~DKASIM
 RLEISFLRTHKLLSSVCSENESEAEADQQMDNLYLKALEGFIAVVTQDGD~~MI~~FLSEN
 25 ISKFMGLTQVELTGHSIFDFTHPCDHEEIRENLSLKNGSGFGKSKDMSTERDFFMR
 MKCTVTNRGRTVNLKSATWKVLHCTGQVKVYNNCPPHNSLCGYKEPLL~~SCLI~~IMCEP
 IQHPSHMDIPLDSKTFLSRHSMDMKFTYCDRITELIGYHPEELLGRSAYEFYHALD
 SENMTKSHQNLCTKGQVVSGQYRMLAKHGGYVWLETQGTVIYNPRNLQPQCIMCVNY
 VLSEIEKNDVVFSMDQTESL~~FKPHLMAMNSIFDSSGKGAVSEKSNFLFTKL~~KEEPEE
 30 LAQLAPTPGD~~AIISLD~~FGNQNFEESSAYGKAILPPSQPWATELRSHSTQSEAGSLPA
 FTV~~PQAA~~PGSTTPSATSSSSSCSTPN~~SPEDYYTSLDNDLKIEVIEKLFAM~~DTEAKD
 Q~~CSTOTDFNELDLETL~~APYIPMDGEDFOLSPICPEERLLAENPOSTPOHCF~~SAM~~TNI
 FOPLAPVAPHSPFLLDK~~FQOOLESKKTEPEHRPMSSIFFDAGSKASLPPCCGOASTP~~
 LSSMGRSNTQWPPDPPLHFGPTKWAVGDORTEFLGAAPLGPPVSPPHVSTFKTRSA
 35 KGFGARGPDVLSPAMVALSNKLKLRQLEYEEQAFQDLSSGDPGGSTSHLMWKRMK

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NLRGGSCPLMPDKPLSANVPNDKFTQNPMRGLGHPLRHLPLPQPPSAISPGENSKSR
 FPPQCYATQYQDYSLSA HKVSGMASRLLGPSFESYLLPELTRYDCEVNVPLGSST
 LLQGGDLLRALDQAT (SEQ ID NO:6)

The N-terminal bHLH domain (which plays a role in DNA
 5 binding) and the C-terminal transactivation domain are
 highlighted (in bold and underlined type, respectively).

Table 5 shows the nucleotide sequence DNA encoding
 human wild type EPAS1. Nucleotides encoding the first
 amino acid of EPAS1 are underlined.

10 Table 5: Nucleotide sequence of human EPAS1 cDNA

```

      1 cctgactgcg cggggcgctc gggacctgcg cgcacctcgg accttcacca ccgccccggg
    61 ccgcgggggag cggacgaggg ccacagcccc ccacccgcca gggagcccag gtgctcggcg
   121 tctgaacgctc tcaaagggcc acagcgacaa tgacagctga caaggagaag aaaaggagta
   181 gctcggagag gaggaaggag aagtccccgg atgctgcgcg gtgccggcgg agcaaggaga
  15 241 cggaggtgtt ctatgagctg gcccatgagc tgcctctgcc ccacagtgtg agtccccatc
   301 tggacaaggc ctccatcatg cgactggaaa tcagcttcct gcgaacacac aagctcctct
   361 cctcagtttg ctctgaaaac gagtccgaag ccgaagctga ccagcagatg gacaacttgt
   421 acctgaaagc cttggagggt ttcatggcgg tggtgaccca agatggcgac atgatcttct
   481 tgtcagaaaa catcagcaag ttcatgggac ttacacaggt ggagctaaca ggacatagta
  20 541 tctttgactt cactcatccc tgcgaccatg aggagattcg tgagaacctg agtctcaaaa
   601 atggctctgg ttttgggaaa aaaagcaaa acatgtccac agagcgggac ttcttcatga
   661 ggatgaagtg caccgtcacc aacagaggcc gtactgtcaa cctcaagtca gccacctgga
   721 aggtcttgca ctgcacgggc caggtgaaag tctacaacaa ctgccctcct cacaatagtc
   781 tgtgtggcta caaggagccc ctgctgtcct gcctcatcat catgtgtgaa ccaatccagc
  25 841 acccatccca catggacatc cccctggata gcaagacctt cctgagccgc cacagcatgg
   901 acatgaagtt cacctactgt gatgacagaa tcacagaact gattggttac caccctgagg
   961 agctgcttgg ccgctcagcc tatgaattct accatgcgct agactccgag aacatgacca
  1021 agagtcacca gaacttgtgc accaagggtc aggtagtaag tggccagtac cggatgctcg
  1081 caaagcatgg gggctacgtg tggctggaga cccaggggac ggcatctac aaccctcgca
  30 1141 acctgcagcc ccagtgcac atgtgtgtca actacgtcct gagtgagatt gagaagaatg
   1201 acgtggtgtt ctccatggac cagactgaat ccctgttcaa gccccacctg atggccatga
   1261 acagcatctt tgatagcagt ggcaaggggg ctgtgtctga gaagagtaac ttcctattca
   1321 ccaagctaaa ggaggagccc gaggagctgg cccagctggc tcccaccca ggagacgcca
   1381 tcatctctct ggatttcggg aatcagaact tcgaggagtc ctcagcctat ggcaaggcca
  35 1441 tcctgcccc gagccagcca tgggccacgg agttgaggag ccacagcacc cagagcgagg
   1501 ctgggagcct gcctgccttc accgtgcccc aggcagctgc cccgggcagc accaccccca
   1561 gtgccaccag cagcagcagc agctgtctca cgcctaatag ccctgaagac tattacacat
   1621 ctttggataa cgacctgaag attgaagtga ttgagaagct cttcgccatg gacacagagg
   1681 ccaaggacca atgcagtacc cagacggatt tcaatgagct ggacttggag acactggcac
  40 1741 cctatatccc catggacggg gaagacttcc agctaagccc catctgcccc gaggagcggc

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1801 tcttggcgga gaaccacag tccaccccc agcactgctt cagtgccatg acaaacatct
1861 tccagccact ggcccctgta gcccgcaca gtcccttctt cctggacaag ttccagcagc
1921 agctggagag caagaagaca gagcccgagc accggcccat gtctccatc ttctttgatg
1981 ccggaagcaa agcatccctg ccaccgtgct gtggccaggc cagcaccctt ctctcttcca
5 2041 tggggggcag atccaatacc cagtggcccc cagatccacc attacatttt gggcccacaa
2101 agtggggcgt cggggatcag cgcacagagt tcttgggagc agcgccgttg gggccccttg
2161 tctctccacc ccatgtctcc accttcaaga caaggtctgc aaagggtttt ggggctcgag
2221 gccagacgt gctgagtccg gccatggtag cctctccaa caagctgaag ctgaagcgac
2281 agctggagta tgaagagcaa gccttcagg acctgagcgg gggggaccca cctggtggca
10 2341 gcacctcaca ttgatgtgg aaacggatga agaacctcag ggggtgggagc tgccctttga
2401 tgccggacaa gccactgagc gcaaagtac ccaatgataa gttcacccaa aaccccatga
2461 ggggcctggg ccatccctg agacatctgc cgctgccaca gcctccatct gccatcagtc
2521 ccggggagaa cagcaagagc aggttcccc cacagtgcta cgccaccag taccaggact
2581 acagcctgtc gtcagccac aaggtgtcag gcatggcaag ccggtgtc gggccctcat
15 2641 ttgagtcta cctgctgccc gaactgacca gatatgactg tgaggtgaac gtgcccgtgc
2701 tgggaagtc cagctcctg caaggagggg acctcctcag agccctggac caggccacct
2761 gagccaggcc ttctacctg gcagcacctc tgccgacgcc gtcccaccag cttcaccc
(SEQ ID NO:7)

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Hypoxia inducible factor-1 α (HIF-1 α) is another member of the PAS family to which EPAS1 belongs. Transcription factors of this family use the bHLH and PAS domains to form heterodimers that subsequently bind to target genes and regulate important biological processes.

EPAS1 plays a role in the regulation of angiogenic factors such as VEGF, VEGF-R such as KDR/flk-1 and flt-1, and Tie2. EPAS1, a nuclear protein with a basic helix-loop-helix (bHLH)/PAS domain, is expressed preferentially in endothelial cells. EPAS1 transcription factor or DNA encoding all or part of EPAS1 (e.g., a fragment containing the C-terminal activation domain) is administered to individuals to promote angiogenesis. To inhibit angiogenesis, EPAS1 antisense sequences are administered to cells to decrease intracellular production of EPAS1 gene product. Administration of DNA encoding an EPAS1-specific antibody (e.g., EPAS1 intrabodies) or EPAS1 dominant negative mutants can also be administered cells to inhibit EPAS1 function, e.g., by inhibiting binding of EPAS1 to cis-acting regulatory sequences of VEGF, VEGF-R, or Tie2 genes or by inhibiting

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EPAS1 transactivation of gene transcription. By regulating transcription of VEGF, VEGF-Rs, and Tie2, EPAS1 is useful to modulate vasculogenesis and angiogenesis.

5 Production of ARNT4-specific antibodies

Anti-ARNT4 antibodies are obtained by techniques well known in the art. Such antibodies can be polyclonal or monoclonal. Polyclonal antibodies are obtained, for example, by the methods described in Ghose et al.,
10 Methods in Enzymology, Vol. 93, 326-327, 1983. For example, a ARNT4 polypeptide, or an antigenic fragment thereof, can be used as an immunogen to stimulate the production of ARNT4-reactive polyclonal antibodies in the antisera of animals such as rabbits, goats, sheep, or
15 rodents. Antigenic polypeptides useful as immunogens include polypeptides which contain a bHLH domain/PAS domain.

Monoclonal antibodies are obtained by standard techniques such as those described by Milstein and Kohler
20 in Nature, 256:495-97, 1975, or as modified by Gerhard, Monoclonal Antibodies, Plenum Press, 1980, pages 370-371. Hybridomas are screened to identify those producing antibodies that are highly specific for an ARNT4 polypeptide. Preferably, the antibody will have an
25 affinity of at least about 10^8 liters/mole and more preferably, an affinity of at least about 10^9 liters/mole.

ARNT4-deficient mice

To further investigate the role of ARNT4 *in vivo*, ARNT4 knockout mice (ARNT4-deficient mice) are generated
30 by homologous recombination. A gene targeting construct for generating ARNT4-deficient mice is made using a targeted gene deletion strategy using standard methods. The deletion in the ARNT4 gene renders the ARNT4 polypeptide non-functional. The linearized targeting
35 construct is transfected into murine D3 embryonic stem

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(ES) cells, and a clone with the correct homologous recombination (yielding the appropriately disrupted ARNT4 gene) is injected into blastocysts and used to generate ARNT4-deficient mice.

5 Activation of the KDR/flk-1 Promoter by EPAS1

EPAS1 and KDR/flk-1 transcripts were found to colocalize in vascular endothelial cells in mouse embryonic and adult tissue. To study the expression of EPAS1 relative to KDR/flk-1, a plasmid containing 4.0 kb
10 of human KDR/flk-1 5'-flanking sequence linked to the luciferase reporter gene and a second vector containing DNA encoding either EPAS1 or another bHLH-PAS domain transcription factor HIF-1 α were cotransfected into bovine aortic endothelial cells (BAEC). EPAS1 but not
15 HIF-1 α markedly increased KDR/flk-1 promoter activity in a dose-dependent manner, and this induction of the KDR/flk-1 promoter by EPAS1 occurred preferentially in endothelial cells. In contrast, both EPAS1 and HIF-1 α activated the VEGF promoter in a non-endothelial cell-
20 specific manner. This is the first demonstration of transactivation of the KDR/flk-1 promoter by EPAS1. By regulating transcription of KDR/flk-1 and VEGF, EPAS1 plays an important role in regulating vasculogenesis and angiogenesis.

25 Cell culture

BAEC were isolated and cultured in DME supplemented with 10% FCS (HyClone, Logan, UT) and antibiotics according to known procedures. BAEC were passed every 3-5 days, and cells from passages 5-7 were
30 used for the transfection experiments. The following cell lines were obtained from the American Type Culture Collection (ATCC) and were cultured in the same medium as BAEC: HeLa cells (human epidermoid carcinoma cells; ATCC #CRL7396 and NIH 3T3 cells (mouse fibroblasts; ATCC
35 #CRL1888).

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RNA isolation and northern analysis

Total RNA was isolated from mouse organs by guanidinium isothiocyanate extraction and centrifugation through cesium chloride according to standard protocols.

5 Total RNA (10 µg) was fractionated on a 1.3% formaldehyde-agarose gel and transferred to Nitropure filters (MSI, Westborough, MA). The filters were then hybridized with ³²P-labeled, randomly primed cDNA probes for 1 h at 68°C in Quick-hyb solution (Stratagene, La

10 Jolla, CA). The hybridized filters were washed in 30 mM NaCl, 3 mM sodium citrate, and 0.1% sodium dodecyl sulfate at 55°C and autoradiographed for 20 h on Kodak XAR film at -80°C. To correct for differences in RNA loading, the filters were rehybridized with a

15 radiolabeled ribosomal 18S-specific oligonucleotide. A 1.8 kb AccI-AccI fragment of mouse EPAS1 (GENBANK Accession # U81983) was used as a probe. The 667 (382-1086) bp mouse KDR/flk-1 cDNA fragment was amplified by the reverse transcriptase PCR by using mouse lung

20 total RNA. The forward (5' GAACTTGGATGCTCTTTGGAAA 3'; SEQ ID NO:8) and reverse (5' CACTTGCTGGCATCATAAGGC 3'; SEQ ID NO:9) primers were used to generate PCR fragments that were subcloned into to a PCR 2.1 vector (Invitrogen, Carlsbad, CA). Nucleotide sequence authenticity was

25 confirmed by the dideoxy chain termination method.

In situ hybridization

To generate probes for in situ hybridization, a 316 (771-1086) bp mouse EPAS1 cDNA and a 342 (2346-2687) bp mouse KDR/flk-1 cDNA from mouse lung total RNA was

30 amplified by reverse transcriptase PCR with the following primers: EPAS1, forward 5' CATCATGTGTGAGCCAATCCA 3' (SEQ ID NO:10) and reverse 5' GTTGTAGATGACCGTCCCCTG 3' (SEQ ID NO:11) KDR/flk-1, forward 5' TGTACTGAGAGATGGGAACCG 3' (SEQ ID NO:12) and reverse 5' CACTTGCTGGCATCATAAGGC 3' (SEQ ID NO:13). PCR fragments were subcloned into the

35

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pCR 2.1 vector in both orientations and the authenticity of the sequences was confirmed.

Slides of E9 mouse sections were purchased from Novagen (Madison, WI). E12 mice and various adult mouse
5 organs were fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Tissue sections (6 μ m thick) were hybridized with a 35 S-UTP-labeled antisense cRNA probe synthesized with the T7 RNA polymerase from linearized
10 plasmids containing appropriate cDNA fragments using standard techniques. As a negative control, tissue sections were also hybridized with 35 S-UTP-labeled sense cRNA probes. After hybridization the tissue sections were washed, and the dried tissue sections were then
15 dipped into Kodak NTB2 emulsion (Eastman Kodak) and exposed for 10-15 days at 4°C. The sections were counterstained with hematoxylin and eosin. Construction of plasmids

pGL2-Basic and pGL2-Control contained the firefly luciferase reporter gene (Promega, Madison, WI). pGL2-
20 Basic had no promoter, whereas pGL2-Control contained the SV40 promoter and enhancer. The pGL2-4kb+296 reporter plasmid was constructed by inserting the human KDR/flk-1 promoter from -4kb to +296 into pGL2-Basic. pVR47/CAT, which contains the human VEGF promoter from -2362 to + 61
25 and the chloramphenicol acetyltransferase (CAT) reporter gene sequence, was also constructed using standard techniques.

The plasmid phEP-1AS was made by cloning the antisense EPAS1 cDNA into pcDNA3. phEP-1 Δ CT, containing
30 a C-terminal deletion mutant of the EPAS1 cDNA, was generated by subcloning a BamHI-XhoI restriction fragment encoding human EPAS1 amino acids 1-690 into pcDNA3. To generate pHIF-1 α , a 2622 bp cDNA fragment containing the entire open reading frame of human HIF-1 α was amplified
35 using human leukocyte total RNA and pfu DNA polymerase

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(Stratagene, La Jolla, CA). The sequences of the forward (5' GTGAAGACATCGCGGGGACC 3'; SEQ ID NO:14) and reverse (5' GTTTGTGCAGTATTGTAGCCAGG 3'; SEQ ID NO:15) primers were based on human HIF-1 α cDNA (Wang et al., 1995, Proc. Natl. Acad. Sci. USA. 92:5510-5514). The PCR fragment was then cloned into pcDNA3, and the sequence was confirmed. Expression of phEP-1, phEP-1 Δ CT, and phHIF-1 α was confirmed by *in vitro* transcription and translation in the TNT-coupled reticulocyte lysate system (Promega, Madison, WI) according to the manufacturer's instructions.

Transient transfection assays

Cells were transfected with 1 μ g of reporter construct and 6 μ g of expression construct by the standard calcium phosphate method. To correct for variability in transfection efficiency against β -galactosidase, 1 μ g of pCMV- β GAL was cotransfected in all experiments. Cell extracts were prepared 48 h after transfection by a detergent lysis method (Promega, Madison, WI). Luciferase activity was measured in duplicate for all samples with an EC&G Autolumat 953 (Gaithersburg, MD) luminometer by the Promega luciferase assay. CAT activity was assayed by a two-phase fluor diffusion method. β -galactosidase activity was assayed using standard methods. The ratio of luciferase or CAT activity to β -galactosidase activity in each sample served as a measure of normalized luciferase or CAT activity. Each construct was transfected at least four times, and each transfection was done in triplicate. Data for each construct are presented as the mean \pm SE.

Statistics

Comparisons between groups were made by a factorial analysis of variance followed by Fisher's least significant difference test when appropriate. Statistical significance was accepted at $p < 0.05$.

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Tissue distribution of EPAS1 and KDR/flk-1 in adult mice

Northern blot analysis was performed with RNA prepared from various adult mouse tissues. EPAS1 mRNA was abundant in the lung, heart, and aorta, organs known to be rich in vascular endothelial cells. When the same blot was hybridized to a mouse KDR/flk-1 probe, the expression pattern of KDR/flk-1 was identical to that of EPAS1. In situ hybridization was performed using an antisense mouse EPAS1 probe to determine which cells in the aorta expressed EPAS1. The EPAS1 message localized to the luminal layer, and the antisense EPAS1 probe but not the sense EPAS1 probe hybridized to the endothelial cells of the aorta.

Tissue distribution of EPAS1 and KDR/flk-1 in developing mouse embryos

To characterize the temporal and spatial patterns of EPAS1 and KDR/flk-1 expression in developing mouse embryos, in situ hybridization was performed with EPAS1 and KDR/flk-1 probes. In embryonic-day (E)9 mice, EPAS1 mRNA was visible in the dorsal aorta, the endocardium of the developing outflow tract, the ventricle, and the perineural vascular plexus. KDR/flk-1 mRNA was expressed similarly in the same organs. At the E9 stage of development, the mouse aorta is composed mainly of a single layer of endothelial cells. Both EPAS1 and KDR/flk-1 were expressed in endothelial cells of the aorta and other organs. At E12.5, EPAS1 mRNA was visible in the intervertebral blood vessels, heart, vascular plexuses in the meninges surrounding both the spinal cord and the brain, and choroid plexus. The distribution of KDR/flk-1 mRNA at E12.5 was strikingly similar. The EPAS1 and KDR/flk-1 mRNAs were both detected in endothelial cells of the blood vessels at higher magnification as well.

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Transactivation of the KDR/flk-1 promoter by EPAS1 in a dose-dependent manner

The colocalization of EPAS1 and KDR/flk-1 indicates that EPAS1 is important in regulating KDR/flk-1 expression. To test the role of EPAS1 in regulation of protein expression, a human EPAS1 expression plasmid (phEP-1) and a reporter plasmid (pGL2-4kb+296) containing approximately 4.0 kb of the human KDR/flk-1 5'-flanking sequence linked to a luciferase reporter gene were cotransfected into BAEC. EPAS1 increased KDR/flk-1 promoter activity in a dose-dependent manner (Fig. 1A). As little as 2 μ g of EPAS1 expression vector phEP-1 increased the promoter activity of KDR/flk-1 by 3-fold, and 6 μ g of the EPAS1 vector increased luciferase activity by 12.9-fold. Upregulation of KDR/flk-1 promoter activity by EPAS1 was specific, since cotransfection of the EPAS1 expression vector had no effect on the activity of pGL2-Control vector driven by the potent SV40 promoter and enhancer.

To identify the EPAS1 domain which participates in transactivation of the KDR/flk-1 promoter, plasmid phEP-1 Δ CT was constructed to express a truncated form of EPAS1 lacking its 180 C-terminal amino acids. Deletion of the 180 C-terminal amino acids of EPAS1 completely abolished its ability to transactivate the KDR/flk-1 promoter (Fig. 1B). These data indicate that the 180 C-terminal amino acids of EPAS1 are necessary for transactivation of the KDR/flk-1 promoter.

These data indicate that induction of the mRNA for KDR/flk-1 colocalizes with that of the mRNA for EPAS1 in vascular endothelial cells from fetal as well as adult mice. EPAS1 also transactivates the promoter of Tie2, which, like KDR/flk-1, is an endothelial cell-specific tyrosine kinase. Expression of Tie2 in endothelial cells is high during fetal development but barely detectable in

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adulthood. In contrast, expression of EPAS1 in endothelial cells is high in fetuses as well as adults. Thus, the target gene for EPAS1 in adults is a VEGF-R such as KDR/flk-1 or flt-1 (as well as VEGF) as evidenced by the data showing that EPAS1 markedly induces KDR/flk-1 promoter activity.

EPAS1 but not HIF-1 α transactivates the KDR/flk-1 promoter preferentially in vascular endothelial cells

To determine whether another member of the BHLH/PAS family transactivated the KDR/flk-1 promoter, the EPAS1 or HIF-1 α expression plasmid and the KDR/flk-1 plasmid pGL2-4kb+296 were cotransfected into BAEC, HeLa cells, and NIH 3T3 cells. EPAS1 expression plasmids in the sense (phEP-1) but not the antisense (phEP-1AS) orientation activated the KDR/flk-1 promoter (Fig. 2A), indicating that the transactivating effect is cell-specific. Although the EPAS1 plasmid markedly increased KDR/flk-1 promoter activity in vascular endothelial cells, it had little effect on KDR/flk-1 promoter activity in HeLa or NIH 3T3 cells (Fig. 2A). HIF-1 α had no effect on KDR/flk-1 promoter activity in all three cell types. The EPAS1 or HIF-1 α expression plasmid was then cotransfected with a reporter plasmid containing the VEGF promoter, pVR47/CAT, to determine whether the differential effects of EPAS1 and HIF-1 α were unique to the KDR/flk-1 promoter. In contrast to its cell-specific effect on the KDR/flk-1 promoter (Fig. 2A), EPAS1 transactivated the VEGF promoter in all three cell types (Fig. 2B). Induction was highest in HeLa cells. Furthermore, HIF-1 α increased VEGF promoter activity in BAEC and HeLa cells (Fig. 2B). These data indicate that the transactivating effect of EPAS1 depends on both the promoter and the cell type.

Although EPAS1 transactivated the KDR/flk promoter preferentially in endothelial cells (Fig. 2A), it

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activated the VEGF promoter in a non-endothelial cell-specific manner (Fig. 2B). Despite the fact that HIF-1 α is 48% homologous to EPAS1, HIF-1 α had no effect on the KDR/flk-1 promoter. In contrast, HIF-1 α transactivated
5 the VEGF promoter. Thus, the effect of EPAS1 on the KDR/flk-1 promoter is specific and cannot be replaced by other members of the PAS family of transcription factors.

EPAS1 heterodimerizes with the aryl hydrocarbon receptor nuclear translocator and transactivates the
10 promoter of Tie2. EPAS1 also markedly increases the promoter activity of KDR/flk-1 and VEGF. Mice deficient in the aryl hydrocarbon receptor nuclear translocator are not viable past E10.5, and the yolk sac shows defective angiogenesis. These data indicate that EPAS1 functions
15 as a nodal transcription factor by regulating expression of VEGF, KDR/flk-1, and Tie2 during vasculogenesis and angiogenesis.

Characterization of functional domains of EPAS1

Functional domains of EPAS1 were identified as
20 follows. The gene encoding VEGF has a cis-acting regulatory sequence to which EPAS1 binds (GCCCTACGTGCTGTCTCA; SEQ ID NO:1) in its 5' flanking region. In cotransfection experiments in BAEC, the EPAS1 expression plasmid activated by 30-fold a CAT reporter
25 plasmid containing 2.3 kb of VEGF 5' flanking sequence (containing SEQ ID NO:1) but not a similar plasmid differing only by a mutation in an amino acid of SEQ ID NO:1. These data indicate that EPAS1 activates the VEGF promoter by binding to DNA containing the sequence of SEQ
30 ID NO:1. To further characterize domains of EPAS1 which function to activate promoters of angiogenic factors in endothelial cells, e.g., the VEGF promoter or VEGF-R promoters, BAEC were cotransfected with expression plasmids encoding EPAS1 mutants and the reporter plasmid.

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Eight mutants were tested. Deletion of the basic region (bHLH region) of EPAS1 (SEQ ID NO:3) completely abolished its ability to induce transcription from the VEGF promoter, indicating that binding of EPAS1 to the cis-acting element though this basic region is critical. Deletion of 180 amino acids from the C-terminus of EPAS1 has little or no effect on the transcriptional transactivation activity of EPAS1 for the VEGF promoter; however, a deletion of the C-terminal 385 amino acids abolished the ability of EPAS1 to activate the VEGF promoter, indicating the presence of a transactivation domain in the portion of EPAS1 spanning amino acids 486-690. Further fine deletion analyses indicated that the transactivation domain of EPAS1 spans amino acids 486-639. An EPAS1 mutant polypeptide lacking the amino acid sequence of SEQ ID NO:2, e.g., an EPAS1 with the amino acid sequence of SEQ ID NO:4, functions as a dominant negative mutant EPAS1 because it inhibited transactivation of the VEGF promoter by wild type EPAS1 in a dose-dependent manner. Deletion analysis is also used to identify domains of EPAS1 which participate in heterodimer formation with ARNT4.

To characterize domains of ARNT4 which function to heterodimerize with EPAS1 and activate promoters of angiogenic factors in endothelial cells, e.g., the VEGF promoter or VEGF-R promoters, BAEC are cotransfected with expression plasmids encoding EPAS1 and ARNT4 deletion mutants and the reporter plasmid as described above. Domains of ARNT4 which participate in EPAS1 heterodimer formation with EPAS1 are identified using the yeast two-hybrid assay or a gel mobility assay. For example, those mutants which fail to activate the VEGF/luciferase promoter cannot form functional dimers with EPAS1.

This assay is also used to identify compounds which inhibit or decrease formation of functional

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ARNT4/EPAS1 heterodimers, and thus, inhibit angiogenesis. In such an assay, expression plasmids which encode wild type or functional fragments of ARNT4 and EPAS1 are cotransfected with a VEGF/luciferase reporter plasmid into an endothelial cell in the presence and absence of a candidate compound. A decrease in the amount of transactivation of the VEGF promoter (e.g., as measured by a standard luciferase assay) in the presence of the candidate compound indicates that the compound inhibits angiogenesis (by inhibiting ARNT4/EPAS1 transactivation of the VEGF promoter).

Generation of a dominant-negative EPAS1 mutants

An adenoviral construct which expresses EPAS1 was generated. Overexpression of EPAS1 dramatically induced VEGF mRNA in human umbilical endothelial cells. In cotransfection experiments, EPAS1 transactivated the VEGF promoter via the HIF-1 binding site. This transactivation was further enhanced by hypoxia. Cotransfection of an aryl hydrocarbon receptor nuclear translocator (ARNT) expression plasmid and EPAS1 expression plasmid synergistically transactivated the VEGF promoter, indicating that heterodimerization of EPAS1 and ARNT is crucial for the transactivation of the VEGF promoter (Fig. 5). Using a gel shift analysis, EPAS1 (but not HIF-1) formed dimers with ARNT4 and bound to the HIF-1 binding site of the VEGF promoter.

Deletion analysis of EPAS1 further defined a potent transactivation domain to span amino acids 486-639 of human EPAS1 (SEQ ID NO:6). The transactivation domain is essential for EPAS1 to transactivate the VEGF promoter. The ability of this domain to activate transcription was confirmed using the GAL4 fusion protein system. Finally, a truncated EPAS1 lacking the transactivation domain (e.g., an EPAS1 polypeptide

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lacking amino acids 486-690 of SEQ ID NO:6 or an EPAS1 polypeptide lacking amino acids 486-639 of SEQ ID NO:6) retained its ability to form heterodimers and to bind the HIF-1 binding site. These data indicate that the mutated

5 EPAS1 polypeptides with lack amino acids in the transactivation domain are dominant negative mutants because they sequester ARNT and prevent the formation of functional EPAS1/ARNT and HIF-1a/ARNT heterodimers. For example, the EPAS1 polypeptide which lacked amino acids

10 486-639 of SEQ ID NO:6 potentially inhibited the induction of the VEGF promoter by EPAS1 and HIF-1a. Transfection of endothelial cells with an adenovirus construct encoding this mutant inhibited VEGF mRNA induction by hypoxia. These results indicate that EPAS1 is an

15 important regulator of VEGF gene expression and that dominant negative EPAS1 mutants (e.g., EPAS1 polypeptides lacking all or part of the transactivation domain (SEQ ID NO:2)) inhibit VEGF promoter activity, and in turn, VEGF expression and angiogenesis.

20 Identification of compounds which modulate EPAS1 binding to cis-regulatory sequences

Modulation of the angiogenesis is achieved by contacting the vascular cells such as vascular endothelial cells with a compound that blocks or enhances

25 EPAS1 binding to cis-acting regulatory sequences of VEGF, VEGF-Rs, or other angiogenic factors in endothelial cells such as Tie2. Such a compound can be identified by methods ranging from rational drug design to screening of random compounds. The latter method is preferable, as

30 simple and rapid assays for testing such compounds are available. Oligonucleotides and small organic molecules are desirable candidate compounds for this analysis.

The screening of compounds for the ability to modulate angiogenesis by affecting EPAS1 transactivation

35 of transcription of angiogenic factors may be carried out

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using in vitro biochemical assays, cell culture assays, or animal model systems. For example, in a biochemical assay, labeled EPAS1 (e.g., EPAS1 labeled with a fluorochrome or a radioisotope) is applied to a column
5 containing immobilized DNA containing the cis-acting regulatory sequence. Alternatively, ARNT4 is immobilized on the column. In this manner, compounds which inhibit ARNT4/EPAS1 heterodimerization may be identified. A candidate compound is applied to the column before,
10 after, or simultaneously with the labeled EPAS1, and the amount of labeled protein bound to the column in the presence of the compound is determined by conventional methods. A compound tests positive for inhibiting EPAS1 binding (thereby having the effect of inhibiting
15 angiogenesis) if the amount of labeled protein bound in the presence of the compound is lower than the amount bound in its absence. Conversely, a compound tests positive for enhancing EPAS1 binding (thereby having the effect of enhancing angiogenesis) if the amount of
20 labeled protein bound in the presence of the compound is greater than the amount bound in its absence. In a variation of the above-described biochemical assay, binding of labeled DNA to immobilized EPAS1 is measured.

As mentioned above, candidate compounds may also
25 be screened using cell culture assays. Cells expressing EPAS1, either naturally or after introduction into the cells of genes encoding EPAS1 are cultured in the presence of the candidate compound. The level of EPAS1 binding in the cell may be inferred using any of several
30 assays. For example, levels of expression of EPAS1 regulated genes (e.g., genes encoding VEGF, VEGF-Rs such as KDR/flk-1 or flt-1) in the cell may determined using, e.g., Northern blot analysis, RNase protection analysis, immunohistochemistry, or other standard methods.

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Compounds identified as having the desired effect, either enhancing or inhibiting EPAS1 binding, can be tested further in appropriate animal models, e.g., an animal with a tumor or atherosclerotic lesion.

- 5 Compounds found to inhibit EPAS1 binding to cis-acting regulatory sequences of genes encoding angiogenic factors may be used in methods for inhibiting pathogenic angiogenesis in order to, e.g., prevent or treat tumor
10 lesion. Compounds found to enhance EPAS1 binding may be used in methods to therapeutically promote new blood vessel formation in adult mammals as discussed above.

The therapeutic compounds identified using the methods of the invention may be administered to a patient
15 by any appropriate method for the particular compound, e.g., orally, intravenously, parenterally, transdermally, transmucosally, by inhalation, or by surgery or implantation at or near the site where the effect of the compound is desired (e.g., with the compound being in the
20 form of a solid or semi-solid biologically compatible and resorbable matrix). Therapeutic doses are determined specifically for each compound, most being administered within the range of 0.001 to 100.0 mg/kg body weight, or within a range that is clinically determined to be
25 appropriate by one skilled in the art.

Identification and molecular cloning of the EPAS1 binding partner, ARNT-4

- Compositions which interact with EPAS1 were identified by screening for endothelial cell proteins
30 which bind to EPAS1. Yeast two hybrid screening of a human umbilical endothelial cell cDNA library was carried out using EPAS1 as a bait. One of the clones isolated encoded a novel bHLH/PAS protein which was found to have similarity with arylhydrocarbon nuclear translocator 3
35 (Arnt3), a member of bHLH/PAS protein which

- 30 -

heterodimerizes with Clock, a gene product involved in regulation of mammalian circadian rhythm. The isolated clone was named ARNT4. As described above, the CLUSTAL W sequence alignment system was used to compare the

5 sequences of ARNT4 with the most closely related known DNA and/or amino acid sequences. With respect to DNA (comparison of coding sequences; untranslated regions excluded), the sequences of hARNT and hARNT4 were found to be 35% identical; the sequences of hBMAL 1b and hARNT4

10 were found to be 56% identical; and the sequences of hARNT and hBMAL 1b were found to be 37% identical. Nucleotide sequence comparisons using the CLUSTAL W system were carried out using the following parameters: KTUP = 2; gap penalty = 5; top diagonals = 4; and window

15 size = 4. With respect to the proteins, the amino acid sequences of hARNT and hARNT4 were found to be 23% identical; the sequences of hBMAL 1b and hARNT4 were found to be 49% identical; and the sequences of hARNT and hBMAL 1b were found to be 26% identical. Amino acid

20 sequence comparisons using the CLUSTAL W system were carried out using the following parameters: KTUP = 1; gap penalty = 3; top diagonals = 5; and window size = 5.

Northern analysis of human tissue revealed that this gene is highly expressed in brain, heart and

25 placenta. In the brain, expression was high in the thalamus and amygdala, an almond-shaped component of the limbic system located in the temporal lobe of the brain.

Expression within human cultured cells demonstrated highest mRNA levels in vascular endothelial

30 cells and smooth muscle cells. ARNT4 was shown to interact with EPAS1 using the yeast two-hybrid assay (Fig. 4). In a gel mobility shift assay using hypoxia responsive element of VEGF gene as the probe, ARNT4 formed a heterodimer with EPAS1 and bound to the hypoxia

35 responsive element of the VEGF gene.

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An expression plasmid encoding EPAS1 and an expression plasmid encoding ARNT4 were cotransfected with a VEGF/luciferase reporter plasmid into bovine aortic endothelial cells. Coexpression of ARNT4 and EPAS1

5 markedly transactivated the VEGF promoter (Fig. 5), and this transactivation was further enhanced by hypoxia. These data indicate that the heterodimer EPAS1/ARNT4 is activated under hypoxic conditions. Taken together, these results indicate that ARNT4, a novel bHLH/PAS

10 protein, is an important regulator of VEGF gene expression especially in vascular system.

Table 6: Human ARNT4 amino acid sequence

	M A A E E	5
	6 E A A A G G K V L R E E N Q C I A P V V	25
15	26 S S R V S P G T R P T A M G S F S S H M	45
	46 T E F P R K R K G S D S D P S Q V E D G	65
	66 E H Q V K M K A F R E A H S Q T E K R R	85
	86 R D K M N N L I E E L S A M I P Q C N P	105
	106 M A R K L D K L T V L R M A V Q H L R S	125
20	126 L K G L T N S Y V G S N Y R P S F L Q D	145
	146 N E L R H L I L K T A E G F L F V V G C	165
	166 E R G K I L F V S K S V S K I L N Y D Q	185
	186 A S L T G Q S L F D F L H P K D V A K V	205
	206 K E Q L S S F D I S P R E K L I D A K T	225
25	226 G L Q V H S N L H A G R T R V Y S G S R	245
	246 R S F F C R I K S C K I S V K E E H G C	265
	266 L P N S K K K E H R K F Y T I H C T G Y	285
	286 L R S W P P N I V G M E E E R N S K K D	305
	306 N S N F T C L V A I G R L Q P Y I V P Q	325
30	326 N S G E I N V K P T E F I T R F A V N G	345
	346 K F V Y V D Q R A T A I L G Y L P Q E L	365
	366 L G T S C Y E Y F H Q D D H N N L T D K	385
	386 H K A V L Q S K E K I L T D S Y K F R A	405
	406 K D G S F V T L K S Q W F S F T N P W T	425
35	426 K E L E Y I V S V N T L V L G H S E P G	445

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446 E A S F L P C S S Q S S E E S S R Q S C 465
 466 M S V P G M S T G T V L G A G S I G T D 485
 486 I A N E I L D L Q R L Q S S S Y L D D S 505
 506 S P T G L M K D T H T V N C R S M S N K 525
 5 526 E L F P P S P S E M G E L E A T R Q N Q 545
 546 S T V A V H S H E P L L S D G A Q L D F 565
 566 D A L C D N D D T A M A A F M N Y L E A 585
 586 E G G L G D P G D F S D I Q W T L 602
 (SEQ ID NO:19)

10

Table 7: Human ARNT4 cDNA

ctccagtc ccgc atgctc agtagctg ctgccggccgggctgcggggcgggcgtccgctg
 cgcgccctacgggctgcgggtggcgccgcgcggcaccgcggcagggcccgccagtc
 cgcttccctgctccagagccgcgcctgggcccggggcagggcgggcccggggctcct
 ccatgctgccagccgcgggctgcggagccgaccaagtggctcctgcg ATG GCG
 15 GCG GAA GAG GAG GCT GCG GCG GGA GGT AAA GTG TTG AGA GAG
 GAG AAC CAG TGC ATT GCT CCT GTG GTT TCC AGC CGC GTG AGT
 CCA GGG ACA AGA CCA ACA GCT ATG GGG TCT TTC AGC TCA CAC
 ATG ACA GAG TTT CCA CGA AAA CGC AAA GGA AGT GAT TCA GAC
 CCA TCC CAA GTG GAA GAT GGT GAA CAC CAA GTT AAA ATG AAG
 20 GCC TTC AGA GAA GCT CAT AGC CAA ACT GAA AAG CGG AGG AGA
 GAT AAA ATG AAT AAC CTG ATT GAA GAA CTG TCT GCA ATG ATC
 CCT CAG TGC AAC CCC ATG GCG CGT AAA CTG GAC AAA CTT ACA
 GTT TTA AGA ATG GCT GTT CAA CAC TTG AGA TCT TTA AAA GGC
 TTG ACA AAT TCT TAT GTG GGA AGT AAT TAT AGA CCA TCA TTT
 25 CTT CAG GAT AAT GAG CTC AGA CAT TTA ATC CTT AAG ACT GCA
 GAA GGC TTC TTA TTT GTG GTT GGA TGT GAA AGA GGA AAA ATT
 CTC TTC GTT TCT AAG TCA GTC TCC AAA ATA CTT AAT TAT GAT
 CAG GCT AGT TTG ACT GGA CAA AGC TTA TTT GAC TTC TTA CAT
 CCA AAA GAT GTT GCC AAA GTA AAG GAA CAA CTT TCT TCT TTT
 30 GAT ATT TCA CCA AGA GAA AAG CTA ATA GAT GCC AAA ACT GGT
 TTG CAA GTT CAC AGT AAT CTC CAC GCT GGA AGG ACA CGT GTG
 TAT TCT GGC TCA AGA CGA TCT TTT TTC TGT CGG ATA AAG AGT
 TGT AAA ATC TCT GTC AAA GAA GAG CAT GGA TGC TTA CCC AAC

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TCA AAG AAG AAA GAG CAC AGA AAA TTC TAT ACT ATC CAT TGC
ACT GGT TAC TTG AGA AGC TGG CCT CCA AAT ATT GTT GGA ATG
GAA GAA GAA AGG AAC AGT AAG AAA GAC AAC AGT AAT TTT ACC
TGC CTT GTG GCC ATT GGA AGA TTA CAG CCA TAT ATT GTT CCA
5 CAG AAC AGT GGA GAG ATT AAT GTG AAA CCA ACT GAA TTT ATA
ACC CGG TTT GCA GTG AAT GGA AAA TTT GTC TAT GTA GAT CAA
AGG GCA ACA GCG ATT TTA GGA TAT CTG CCT CAG GAA CTT TTG
GGA ACT TCT TGT TAT GAA TAT TTT CAT CAA GAT GAC CAC AAT
AAT TTG ACT GAC AAG CAC AAA GCA GTT CTA CAG AGT AAG GAG
10 AAA ATA CTT ACA GAT TCC TAC AAA TTC AGA GCA AAA GAT GGC
TCT TTT GTA ACT TTA AAA AGC CAA TGG TTT AGT TTC ACA AAT
CCT TGG ACA AAA GAA CTG GAA TAT ATT GTA TCT GTC AAC ACT
TTA GTT TTG GGA CAT AGT GAG CCT GGA GAA GCA TCA TTT TTA
CCT TGT AGC TCT CAA TCA TCA GAA GAA TCC TCT AGA CAG TCC
15 TGT ATG AGT GTA CCT GGA ATG TCT ACT GGA ACA GTA CTT GGT
GCT GGT AGT ATT GGA ACA GAT ATT GCA AAT GAA ATT CTG GAT
TTA CAG AGG TTA CAG TCT TCT TCA TAC CTT GAT GAT TCG AGT
CCA ACA GGT TTA ATG AAA GAT ACT CAT ACT GTA AAC TGC AGG
AGT ATG TCA AAT AAG GAG TTG TTT CCA CCA AGT CCT TCT GAA
20 ATG GGG GAG CTA GAG GCT ACC AGG CAA AAC CAG AGT ACT GTT
GCT GTC CAC AGC CAT GAG CCA CTC CTC AGT GAT GGT GCA CAG
TTG GAT TTC GAT GCC CTA TGT GAC AAT GAT GAC ACA GCC ATG
GCT GCA TTT ATG AAT TAC TTA GAA GCA GAG GGG GGC CTG GGA
GAC CCT GGG GAC TTC AGT GAC ATC CAG TGG ACC CTC
25 tagcctttgatttttaactccaaaaatgagaaacatttttaaagcattatttacgaaa
aaactgtctcaactattcttaagtactgtattgatattgtttgtatcttttattaat
gttctaccactttttatagatttgcattctcctgtcacagggatgtggggaaatacg
ttttcctcccaagagaaccaagtttattatagactcctttattcagtgaaatggctt
ataatccactagttgccatatttttgctaaaatatttctaaccaagaatactactta
30 catattgttttggctttgttttatattttgatgcagtttttttagttgaggtaatgt
aatatattgatgttttcctttgtgtctaagattgatttataatagtaggtttgtata
atttggaaacattttccatgccttgccaatttccttaattgaggatagggcttacaca
ctttaagaaaacagtgagtacttgaacattttaaaggacagtgcaatttatagtcac
aatcacattgaatactgtattttgatctttggagacttaggcaagcacagagctggga
35 tatttatgctcagttgagcactttaagatgaattttaagtgagatgatttcttgctt

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aaaactcagaaagtcaaaagagtttcagctttccttacagaaaaggaaggatccttgg
gccctagatccttggggattaacctctgcatataagatttactcttaataggccagac
gtggtgctcacgcctgtaatcccagtaacttgggaggctgagacgggcagatcactt
gaggtcaggagttcaagaccagcctggccaatatggtgaaaccccgtttctactaaa
5 aatacaaaaaaaattacccaggcactcactccttgaggttaactaaccaactcccacga
taatgacagtccattcatgagcgcaaaggcctcatgacctaattggcacacacctgta
atcccaactgcttgggaggctgagggcagaggattgcttgaacctgggaggcagagg
ttgcagtgagccgagatcgaccactgcactccagtctgggcaacagagtgagactt
catctcaaaaaaagtaaaaaaaagatttaataataatcactgaagatctctattata
10 gatagattagggtttttgacattggaaacatacttagggatagatttgtcctaaagga
aaaaagtagggccgggcagattaaatgtcttgtgttaaagtcacacattaaattcagt
cacacattaaattcatagagttttaaatgtttaatgtatataaaccagtttctttat
acacatttgggaaaacattggtctcacagattaaatgattaactaactgaccagga
actagttgtagctttctaagtaattaggcaattacagttattgcctgtaaccaaagg
15 taataaaaacaaaatgacaagtacatgtttaaaattatgaggcaatgagaaataattt
aaaaaccaattttctagttataattttaaatttggagagcatttttaacagtaatta
atccagaggtggctcaaattgagtataagaattaagattattttaaatactgcatgt
ctaccttctcggggatcatactttataacactttctgcttcagtagctcttcatagc
ttgccaagtatgctcccatattttctctctcgtgcctcgcaaatgaaagtcatagag
20 gctgggaactcatggggcagccctcagacttcaatgtgggcttcaaaccagtttcc
tgttctatatggtgctacatctttccagaaaatttccctcagagccctcgccaaaa
caaagcattattttgaccctgcatgctatttcttttagctgtagggtgatagattagaa
cttctgtcagacatgttaatgacaaacataccaacagacaataaccaaagcaaatgt
ttccttcaagtgtgaaatgtgcaggggctcgtgggcaaggatgtattggcacactgt
25 cctcttgaactgatagtgctccagcaatgttggagggttggcaccattcctggtccga
cacttgaggacctgagagacatcaggtttagaatgagccaaagaaatcctacaagat
ggggagaattggtgtgcagcagcctaagtgttatagttaagtctaagaagtatgaa
agatccccctgtgttctctaaattgagcagaggggcctgcctaccaatatcacttttt
aggggactgaaccattgcaggttagacttggcttccaaagagtctgcctaagccagg
30 ggtggcagggtaggcatcatagctggatggcctcaaaagcagatgggggcagactt
gccctcgtgatgccaggatttgagaggcagagtttctagaggagaccagtgtgcc
tctcacagtggcagttttttctctttgcaagaggaggggctgttcaattccatagac
cagtgggcagatagccagttgaatactctgtgcatgggttgatcctttattagttcg
ctctaataattttctgtagatccttttgcctggactcaaaatctaatacatgcatt
35 gtatgataccgtagctctcctaagggttgtgtttccttcaaaatgttttagttttct

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tcaactaaatttgatttttgcgtgtagaagtgacatatttttatggtatacactatg
ttccttttttctactgcgagtcattttttgaattttcgtgagaaagaatatatcta
caaattgcacgaaagtatcataaaaacagtactctagagcagcgctgtccaatagaa
atataatctgagccacatgtataattttattttcttctagccacattaaagaagtaa
5 aaagatacaagtagaactaatttttaattggttttaattcagtatatccaaaatatcatt
tgaacatgtaattaataataaaattattaatgtgatattttacattcttttggttaata
ctagtcttcaaaatctggtatgtatcttacattgatagcacatctcactttgtacta
gccacattgcaagtgcctcagtagccacatgtggctagtggctactgcactggacagc
acagttctaggttccaccctaacacccaagtccgtggattagaatcccagaatcag
10 agctggaagtaaacatagagatcaaacctccttttaaaaatgaggacgctgaggcac
agagtttaaatggcttgcatgaggtcatacagctaaattcagcctcaacagggctctt
ctgattccaggcactcttcccactccactacattactgtagtggtaattcttaggggt
taaaaaaagtgtagagttaggccggcgagtggtcatgcctgtaatcccagcactt
tgggaggccgaagtgggaggatcacgaggtcaggagatcgagaccatcctggccaac
15 atggtgaaaccccgtctctactgaaaatacaaagcaaaattagccaggtgtggtggc
gggcgcctgtggtcccagctgctctggaggctgaggcagaatggcgtgaaccaggga
ggcagagatggcagtgagccaagatcgcgccactgcaccccagcctgggcgacagag
cgagactccatctcaaaaaaaaaaaaaaaaaaaaaaagaaaagaaaagaaagtctag
agaacattatattaagtgggttattattgaagtagaccaaagtttataccataaggat
20 atttttccttaataacatgtttgaagaacaattatttattgatccttgaatctgta
agatcaaataacaagtctctatccatgttaccaaatttaaccttttgaaaataataa
actttaaaatatcagatgtgttattacaggatgatacttggaatcaagtgaatgag
ttatatggtcatcactaaatttagaaatctattgtgaaacaaagacaaacaggaaag
tacagaatagagacttttagtaataaatggaatttaaaagaaagtgtttatttaca
25 gtgtcacgacagaaaaggatgtctttgtgtcatagtctttgagggatctccgtaaa
atctggggcacaggtacaagaaatagccaatattttagttcccagaccatgtttagta
gtgtccagtttcagatcatgctgccaaagggtatctccccctcaggtgggtcatcac
tgagccctggaattggagactcatacttgcccagcacaaatgttacgggcagacaggc
cgacatctatgattagctagaagccataaaagaaaagctgctaagtggccactagggtg
30 ccacttttctgtttttgtaatgctttcattagcagatctttttttccaagctccat
ggggcctatgagaggcatttatgatttttgtgcctacaataagtgcgctgtctggt
gtgagttgttttatgagaaatgctttccaagggaggtctaggaagatcctgacacat
aagaactttggcttagagagctttccaggtgtagtgccaaataaaaaactgacctggaa
agaaaacctgcccagcacggaacatgctttctgaactcacttgagagtgatggtgt
35 atgtcacttctcatatattcttgagtttagatttgtcttttatacaatttttagctc

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ttttccagttcacttgtgctcgtctgtatattggtattttttaaatTTTTGTGGTAA
 taatgaaaagagtgaattatattttataattactcattttagtTTTTTTTTTTAA
 ttttaataaacttcctccaaaaagtgtcccttaaaa (SEQ ID NO:20)

ARNT4 coding sequence in Table 7 is indicated by upper
 5 case letters (nucleotides 220 to 2025) with the
 termination codon underlined.

Diagnosis and treatment of circadian rhythm disorders

ARNT4 is involved in regulating circadian rhythm,
 e.g., by forming a heterodimer with Clock, a protein that
 10 regulates the timing of fatigue and alertness.
 Individuals with circadian rhythm disorders are screened
 for mutations in the an ARNT4 gene product or ARNT4 gene,
 e.g., by detecting restriction fragment length
 polymorphisms (RFLPs) or by PCR. Individuals with
 15 symptoms of circadian rhythm disorders and identified as
 having a mutated ARNT4 gene are treated by administering
 DNA encoding a normal ARNT4 gene product. For example,
 DNA containing the coding sequence of SEQ ID NO:20 is
 administered to such individuals using standard gene
 20 therapy techniques described herein. Similarly, an
 abnormally low or high level of ARNT4 protein or
 transcript is detected in an individual suffering from
 such disorders, the levels can be normalized by antisense
 therapy to inhibit ARNT4 production or gene therapy to
 25 augment production. ARNT4 levels may also be altered to
 artificially regulate circadian rhythm, e.g., to induce
 long periods of sleep in patients to improve the healing
 process or in individuals travelling long distances such
 astronauts during space travel.

30 Antisense Therapy

Nucleic acids complementary to all or part of the
 human EPAS1 cDNA (GenBank Accession # U81984; SEQ ID
 NO:7) may be used in methods for antisense treatment to
 inhibit expression of EPAS1. Nucleic acids complementary
 35 to all or part of the human ARNT4 cDNA (SEQ ID NO:20) may

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be used in methods for antisense treatment to inhibit expression of ARNT4. Antisense treatment may be carried out by administering to a mammal, such as a human, DNA containing a promoter, e.g., an endothelial cell-specific promoter, operably linked to a DNA sequence (an antisense template), which is transcribed into an antisense RNA. Alternatively, as mentioned above, antisense oligonucleotides may be introduced directly into vascular cells. The antisense oligonucleotide may be a short nucleotide sequence (generally at least 10, preferably at least 14, more preferably at least 20 (e.g., at least 30), and up to 100 or more nucleotides) formulated to be complementary to a portion, e.g., the coding sequence, or all of EPAS1 mRNA or ARNT4 mRNA. For example, the sequence is complementary some or all of the C-terminal activation domain; alternatively, the sequence may be complementary to all or part of the N-terminal DNA binding domain. The antisense sequence is complementary to DNA encoding residues 75 to 128, inclusive, of SEQ ID NO:19; the antisense sequence. Alternatively, the antisense sequence is complementary to DNA encoding residues 155 to 207, inclusive, of SEQ ID NO:19, or encoding residues 232 to 384 of SEQ ID NO:19.,

Standard methods of administering antisense therapy have been described (see, e.g., Melani et al., 1991, Cancer Res. 51:2897-2901). Following transcription of a DNA sequence into an antisense RNA, the antisense RNA binds to its target nucleic acid molecule, such as an mRNA molecule, thereby inhibiting expression of the target nucleic acid molecule. For example, an antisense sequence complementary to a portion or all of EPAS1 mRNA could be used to inhibit the expression of EPAS1, thereby decreasing the level of transcription of angiogenic factors such as VEGF or VEGF-Rs, which in turn leads to a decrease in new blood vessel formation. Oligonucleotides

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complementary to various portions of EPAS1 mRNA or ARNT4 mRNA can readily be tested in *in vitro* for their ability to decrease production of their respective gene products, using assays similar to those described herein.

- 5 Sequences which decrease production of EPAS1 message or ARNT4 message *in vitro* cell-based or cell-free assays can then be tested *in vivo* in rats or mice to determine whether blood vessel formation is decreased.

- Preferred vectors for antisense templates are
- 10 viral vectors, including those derived from replication-defective hepatitis viruses (e.g., HBV and HCV), retroviruses (see, e.g., WO 89/07136; Rosenberg et al., 1990, N. Eng. J. Med. 323(9):570-578), adenovirus (see, e.g., Morsey et al., 1993, J. Cell. Biochem., Supp.
- 15 17E,)), adeno-associated virus (Kotin et al., 1990, Proc. Natl. Acad. Sci. USA 87:2211-2215,)), replication defective herpes simplex viruses (HSV; Lu et al., 1992, Abstract, page 66, Abstracts of the Meeting on Gene Therapy, Sept. 22-26, Cold Spring Harbor Laboratory, Cold
- 20 Spring Harbor, New York), and any modified versions of these vectors. The invention may utilize any other delivery system which accomplishes *in vivo* transfer of nucleic acids into eucaryotic cells. For example, the nucleic acids may be packaged into liposomes, receptor-
- 25 mediated delivery systems, non-viral nucleic acid-based vectors, erythrocyte ghosts, or microspheres (e.g., microparticles; see, e.g., U.S. Patent No. 4,789,734; U.S. Patent No. 4,925,673; U.S. Patent No. 3,625,214; Gregoriadis, 1979, Drug Carriers in Biology and Medicine,
- 30 pp. 287-341 (Academic Press,)). Alternatively, naked DNA may be administered. Delivery of nucleic acids to a specific site in the body for antisense therapy may also be accomplished using a biolistic delivery system, such as that described by Williams et al., 1991, Proc. Natl.
- 35 Acad. Sci. USA 88:2726-2729.

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Antisense oligonucleotides may consist of DNA, RNA, or any modifications or combinations thereof. As an example of the modifications that the oligonucleotides may contain, inter-nucleotide linkages other than

5 phosphodiester bonds, such as phosphorothioate, methylphosphonate, methylphosphodiester, phosphorodithioate, phosphoramidate, phosphotriester, or phosphate ester linkages (Uhlman et al., 1990, Chem. Rev. 90(4):544-584; Anticancer Research, 1990, 10:1169) may be

10 present in the oligonucleotides, resulting in their increased stability. Oligonucleotide stability may also be increased by incorporating 3'-deoxythymidine or 2'-substituted nucleotides (substituted with, e.g., alkyl groups) into the oligonucleotides during synthesis, by

15 providing the oligonucleotides as phenylisourea derivatives, or by having other molecules, such as aminoacridine or poly-lysine, linked to the 3' ends of the oligonucleotides e.g., Anticancer Research, 1990, 10:1169-1182). Modifications of the RNA and/or DNA

20 nucleotides may be present throughout the oligonucleotide, or in selected regions of the oligonucleotide, e.g., in the 5' and/or 3' ends. The antisense oligonucleotides may also be modified so as to increase their ability to penetrate the target tissue by,

25 e.g., coupling the oligonucleotides to lipophilic compounds. Antisense oligonucleotides based on the human EPAS1 nucleotide sequence (SEQ ID NO:7) or the human ARNT4 nucleotide sequence (SEQ ID NO:20) can be made by any method known in the art, including standard chemical

30 synthesis, ligation of constituent oligonucleotides, and transcription of DNA complementary to the all or part of the EPAS1 cDNA or ARNT4 cDNA.

EPAS1 is naturally expressed in vascular endothelial cells. These cells are, therefore, the

35 preferred cellular targets for antisense therapy.

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Targeting of antisense oligonucleotides to endothelial cells is not critical to the invention, but may be desirable in some instances, e.g. systemic administration of antisense compositions. Targeting may be achieved, 5 for example, by coupling the oligonucleotides to ligands of endothelial cell surface receptors. Similarly, oligonucleotides may be targeted to endothelial cells by being conjugated to monoclonal antibodies that specifically bind to endothelial-specific cell surface 10 proteins. Antisense compositions may also be administered locally, e.g., at the site of an atherosclerotic lesion or at the site of a tumor.

Therapeutic applications of antisense oligonucleotides in general are described, e.g., in the 15 following review articles: Le Doan et al., Bull. Cancer 76:849-852, 1989; Dolnick, Biochem. Pharmacol. 40:671-675, 1990; Crooke, Annu. Rev. Pharmacol. Toxicol. 32, 329-376, 1992. Antisense nucleic acids may be used alone or combined with one or more materials, including other 20 antisense oligonucleotides or recombinant vectors, materials that increase the biological stability of the oligonucleotides or the recombinant vectors, or materials that increase the ability of the therapeutic compositions to penetrate endothelial cells selectively.

25 Therapeutic compositions, e.g., inhibitors of EPAS1 and/or ARNT4 transcription or transactivating function, may be administered in pharmaceutically acceptable carriers (e.g., physiological saline), which are selected on the basis of the mode and route of 30 administration and standard pharmaceutical practice. Suitable pharmaceutical carriers, as well as pharmaceutical necessities for use in pharmaceutical formulations, are described in Remington's Pharmaceutical Sciences, a standard reference text in this field, and in 35 the USP/NF. The compound may be administered with

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intravenous fluids as well as in combination with other anti-inflammatory agents, e.g., antibiotics; glucocorticoids, such as dexamethasone (Dex), or other chemotherapeutic drugs for the treatment of

5 atherosclerotic lesions and tumors, respectively.

A therapeutically effective amount is an amount which is capable of producing a medically desirable result in a treated animal. As is well known in the medical arts, dosage for any one patient depends upon

10 many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. Dosages will vary, but a preferred dosage for intravenous

15 administration of DNA is approximately 10^6 to 10^{22} copies of the DNA molecule. The compositions of the invention may be administered locally or systemically. Administration will generally be parenterally, e.g., intravenously. As mentioned above, DNA may also be

20 administered directly to the target site, e.g., by biolistic delivery to an internal or external target site or by catheter to a site in an artery.

Gene Therapy

Compositions which enhance intracellular

25 production of EPAS1 (or its binding to a cis-acting regulatory region of a gene encoding VEGF or a VEGF-R) or ARNT4 may be used in methods to promote new blood vessel formation, e.g., to promote angiogenesis in wound healing (e.g., healing of broken bones, burns, diabetic ulcers,

30 or traumatic or surgical wounds) and organ transplantation. Such compounds may be used to treat peripheral vascular disease, cerebral vascular disease, hypoxic tissue damage (e.g., hypoxic damage to heart tissue), or coronary vascular disease as well as to treat

35 patients who have, or have had, transient ischemic

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attacks, vascular graft surgery, balloon angioplasty, frostbite, gangrene, or poor circulation.

Since EPAS1 and ARNT4 are nuclear proteins, a preferred method of increasing the levels of these proteins or polypeptides in a cell (to increase transcription of such angiogenic factors as VEGF or VEGF-Rs) is intracellular expression of recombinant EPAS1 or ARNT4 or active fragments thereof, e.g., transactivating fragments. DNA encoding EPAS1 or ARNT4 is administered alone or as part of an expression vector as described above. The DNA introduced into its target cells, e.g., endothelial cells at an anatomical site in need of angiogenesis, directs the production of recombinant EPAS1 or ARNT4 or fragments thereof in the target cell, to enhance production of new blood vessels. For inhibition of angiogenesis, gene therapy are also used to introduce administer DNA encoding a dominant negative mutant of EPAS1 such as DNA encoding a polypeptide with the amino acid sequence of SEQ ID NO:4 or a polypeptide with the amino acid sequence of residues 486-639 of SEQ ID NO:6.

Antibodies and intrabodies

Anti-EPAS1 antibodies were obtained using techniques well known in the art. Such antibodies can be polyclonal or monoclonal. Polyclonal antibodies can be obtained, for example, by the methods described in Ghose et al., Methods in Enzymology, Vol. 93, 326-327, 1983. An EPAS1 polypeptide, or an antigenic fragment thereof, was used as the immunogen to stimulate the production of EPAS1-reactive polyclonal antibodies in the antisera of animals such as rabbits, goats, sheep, rodents and the like. EPAS1-specific antibodies were raised by immunizing animals with a C-terminal EPAS1 polypeptide spanning amino acids 668-829 of human EPAS (PGGSTSHLMWKRMKNLRGGSCPLMPDKPLSANVPNDKFTQNP MRGL

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HPLRHLPLPQPPSAISPGENSKSRFPPQCYATQYQDYSLSAHKVSGMASRLLGP;
 (SEQ ID NO:17) and a C-terminal EPAS1 polypeptide spanning
 amino acids 641-875 of mouse EPAS1 DPPLHFGPTKWPVGDQSAE
 SLGALPVGSWQLELPSAPLHVSMFKMRSKDFGARGPYMMSPAMIALSNK
 5 LKLKRQLEYEEQAFQDTSGGDPPGTSSSHLMWKRMKSLMGGTCPLMPDKT
 ISANMAPDEFTQKSMRGLGQPLRHLPPPQPPSTRSSGENAKTGFPQCYA
 SQFQDYGPPGAQKVSGVASRLLGPSFEPYLLPELTRYDCEVNVVPVPGSST
 LLQGRDLLRALDQAT (SEQ ID NO:18).

Monoclonal antibodies are obtained by the process
 10 described by Milstein and Kohler in Nature, 256:495-97,
 1975, or as modified by Gerhard, Monoclonal Antibodies,
 Plenum Press, 1980, pages 370-371. Hybridomas are
 screened to identify those producing antibodies that are
 highly specific for an EPAS1 polypeptide. Preferably,
 15 the antibody will have an affinity of at least about 10^8
 liters/mole and more preferably, an affinity of at least
 about 10^9 liters/mole. Monoclonal antibodies can be
 humanized by methods known in the art, e.g, MAbs with a
 desired binding specificity can be commercially humanized
 20 (Scotgene, Scotland; Oxford Molecular, Palo Alto, CA).

Following identification of a hybridoma producing
 a suitable monoclonal antibody, DNA encoding the antibody
 is cloned. DNA encoding a single chain EPAS1-specific
 antibody in which heavy and light chain variable domains
 25 (separated by a flexible linker peptide such as Gly₄-Ser₃,
 (SEQ ID NO:16) is cloned into an expression vector using
 known methods (e.g., Marasco et al., 1993, Proc. Natl.
 Acad. Sci. USA 90:7889-7893 and Marasco et al., 1997,
 Gene Therapy 4:11-15). Such constructs are introduced
 30 into cells, e.g., using gene therapy techniques described
 herein, for intracellular production of the antibodies.
 Intracellular antibodies, i.e., intrabodies, are used to
 inhibit binding of endogenous EPAS1 to its target DNA
 (e.g., cis-acting regulatory sequences of genes encoding
 35 VEGF or VEGF-Rs), which in turn, decreases production of

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- these angiogenic factors and decreases new blood vessel formation in the treated mammal. Intrabodies which bind to a C-terminal transactivation domain of EPAS1 inhibit the ability of EPAS1 to induce transcription of a gene
- 5 encoding an angiogenic factor such as VEGF or a VEGF-R. A similar strategy is used to make intrabodies which bind to intracellular ARNT4. Such intrabodies bind to ARNT4 and prevent heterodimerization with EPAS1, and as a result, inhibit transactivation of the VEGF promoter.
- 10 Inhibition of VEGF promoter activity, in turn, leads to inhibition of new blood vessel formation.

Other embodiments are within the following claims.

What is claimed is:

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1. A method of inhibiting angiogenesis in a mammal comprising administering to said mammal a compound which inhibits binding of endothelial PAS domain protein-1 (EPAS1) to cis-acting transcription regulatory DNA of
5 an angiogenic factor.

2. The method of claim 1, wherein said angiogenic factor is a vascular endothelial growth factor receptor (VEGF-R).

3. The method of claim 2, wherein said receptor
10 is KDR/flk-1.

4. The method of claim 2, wherein said receptor is flt-1.

5. The method of claim 1, wherein said angiogenic factor is vascular endothelial growth factor (VEGF).

15 6. The method of claim 1, wherein said angiogenic factor is Tie2.

7. The method of claim 1, wherein said compound inhibits transcription of said angiogenic factor.

8. The method of claim 1, wherein said regulatory
20 DNA comprises GCCCTACGTGCTGTCTCA (SEQ ID NO:1).

9. The method of claim 1, wherein said compound is an EPAS1 polypeptide lacking a transactivation domain.

10. The method of claim 9, wherein said transactivation domain comprises the amino acid sequence
25 of SEQ ID NO:2.

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11. The method of claim 9, wherein said transactivation domain comprises the amino acids 486-639 of SEQ ID NO:6.

12. The method of claim 9, wherein said
5 polypeptide comprises the amino acid sequence of SEQ ID NO:4.

13. The method of claim 1, wherein said compound is a nucleic acid encoding an EPAS1 polypeptide lacking the amino acid sequence of SEQ ID NO:2.

10 14. The method of claim 1, wherein said compound is a nucleic acid encoding an EPAS1 polypeptide lacking amino acids 486-639 of SEQ ID NO:6.

15 15. The method of claim 1, wherein said compound is a antisense nucleic acid molecule comprising at least 10 nucleotides, wherein the sequence of said molecule is complementary to part of or all of an mRNA encoding EPAS1 polypeptide.

16. The method of claim 1, wherein said compound is an EPAS1-specific intrabody.

20 17. The method of claim 1, wherein said compound is administered to a site of an atherosclerotic lesion in said mammal.

18. The method of claim 1, wherein said compound is administered to a tumor site in said mammal.

25 19. An antibody which binds to EPAS1.

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20. The antibody of claim 19, wherein said antibody binds to a C-terminal activation domain of EPAS1.

21. The antibody of claim 20, wherein said
5 activation domain comprises SEQ ID NO:2.

22. A method of promoting angiogenesis in a mammal comprising administering to said mammal a compound which increases expression of VEGF or a VEGF-R in an endothelial cell.

10 23. The method of claim 19, wherein said VEGF-R is KDR/flk-1 or flt-1.

24. A substantially pure DNA comprising a sequence encoding a aryl hydrocarbon receptor nuclear translocator-4 (ARNT4) polypeptide.

15 25. The DNA of claim 24, wherein said DNA encodes a human ARNT4 polypeptide.

26. The DNA of claim 24, wherein said polypeptide comprises the amino acid sequence of residues 75 to 128, inclusive, of SEQ ID NO:19.

20 27. The DNA of claim 24, wherein said polypeptide comprises the amino acid sequence of residues 155 to 207, inclusive, of SEQ ID NO:19.

28. The DNA of claim 24, wherein said polypeptide comprises the amino acid sequence of residues 232 to 384,
25 inclusive, of SEQ ID NO:19.

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29. A substantially pure DNA comprising a nucleotide sequence having at least 50% sequence identity to SEQ ID NO:20.

30. The DNA of claim 24, wherein said DNA
5 comprises the coding sequences of SEQ ID NO:20.

31. The DNA of claim 24, wherein said polypeptide comprises the amino acid sequence of SEQ ID NO:19.

32. A substantially pure DNA comprising a strand which hybridizes at high stringency to a strand of DNA
10 consisting of the coding sequence of SEQ ID NO:20, or the complement thereof.

33. A substantially pure DNA comprising a sequences at least 50% sequence identity to the coding sequence of SEQ ID NO:20, and encoding a polypeptide
15 having the biological activity of an ARNT4 polypeptide.

34. A substantially pure ARNT4 polypeptide.

35. The polypeptide of claim 34, wherein said polypeptide is human ARNT4.

36. The polypeptide of claim 34, wherein said
20 polypeptide comprises the amino acid sequence of residues 75 to 128, inclusive, of SEQ ID NO:19.

37. The polypeptide of claim 34, wherein said polypeptide comprises the amino acid sequence of residues 155 to 207, inclusive, of SEQ ID NO:19.

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38. The polypeptide of claim 34, wherein said polypeptide comprises the amino acid sequence of residues 232 to 384, inclusive, of SEQ ID NO:19.

39. The polypeptide of claim 34, wherein said
5 polypeptide comprises the amino acid sequence of SEQ ID NO:19.

40. The polypeptide of claim 34, wherein said polypeptide comprises an amino acid sequence at least 50% identical to SEQ ID NO:19.

10 41. The polypeptide of claim 34, wherein said polypeptide comprises the amino acid sequence of SEQ ID NO:19.

42. A vector comprising the DNA of claim 24.

43. A host cell comprising the DNA of claim 24.

15 44. A transgenic non-human animal the germ cells and nucleated somatic cells of which comprise a null mutation in a gene encoding ARNT4.

45. A method of inhibiting angiogenesis in a mammal comprising administering to said mammal a compound
20 which inhibits binding of EPAS1 to ARNT4.

46. The method of claim 45, wherein said compound is an ARNT4 polypeptide.

47. The method of claim 45, wherein said polypeptide comprises the amino acid sequence of residues
25 75 to 128, inclusive, of SEQ ID NO:19.

- 50 -

48. The method of claim 45, wherein said polypeptide comprises the amino acid sequence of residues 155 to 207, inclusive, of SEQ ID NO:19.

49. The method of claim 45, wherein said
5 polypeptide comprises the amino acid sequence of residues 232 to 384, inclusive, of SEQ ID NO:19.

50. The method of claim 45, wherein said polypeptide comprises the amino acid sequence of SEQ ID NO:19.

10 51. An EPAS1 polypeptide lacking a transactivation domain.

52. The polypeptide of claim 51, wherein said transactivation domain comprises the amino acid sequence of SEQ ID NO:2.

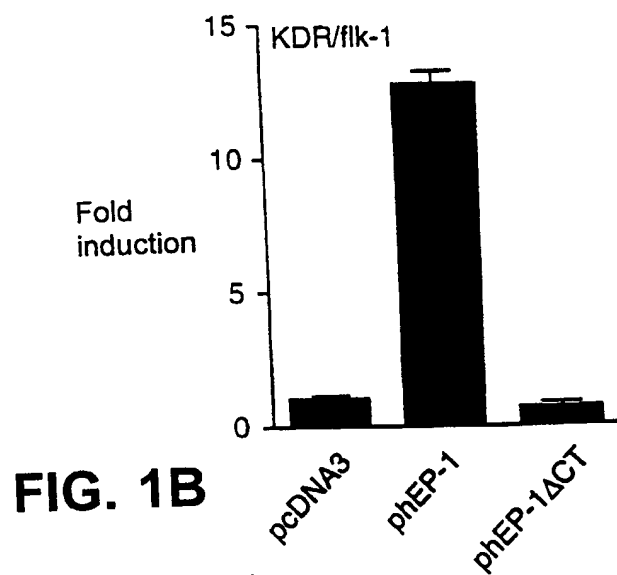
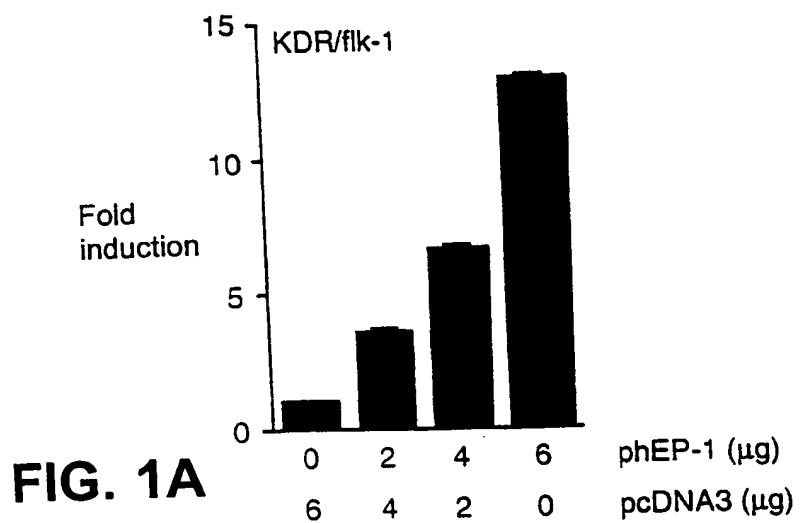
15 53. The polypeptide of claim 51, wherein said transactivation domain comprises the amino acids 486-639 of SEQ ID NO:6.

54. The polypeptide of claim 51, wherein said polypeptide comprises the amino acid sequence of SEQ ID
20 NO:4.

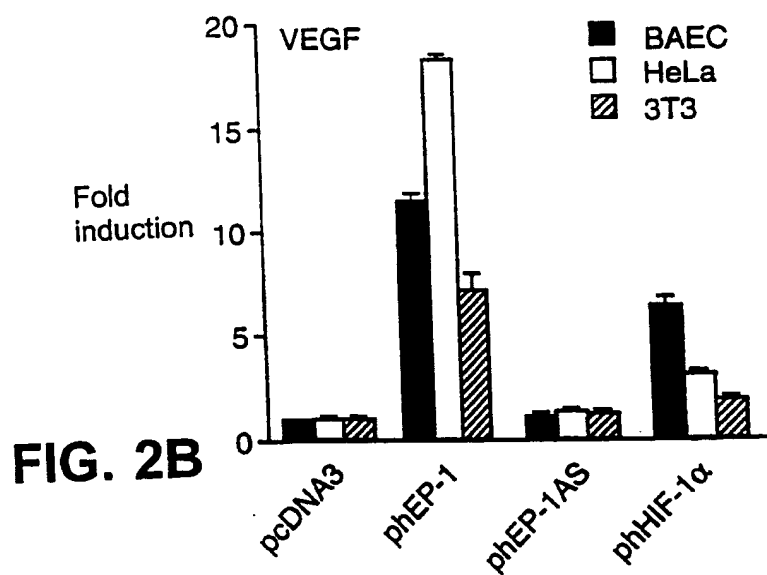
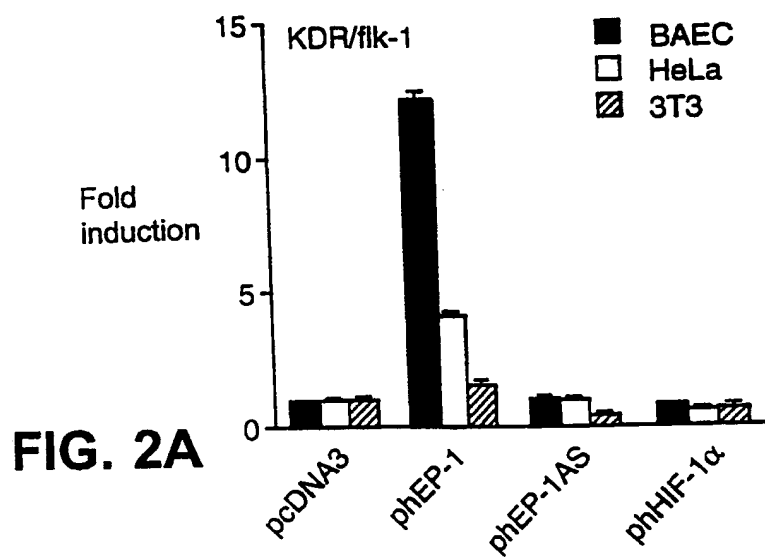
55. A nucleic acid encoding an EPAS1 polypeptide lacking the amino acid sequence of SEQ ID NO:2.

56. The nucleic acid of claim 55, wherein said nucleic acid encodes an EPAS1 polypeptide lacking amino
25 acids 486-639 of SEQ ID NO:6.

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1	MAAEE E A A G G K V L R E E N Q C I A P V V S S R V S P G T R P T A M G S F S S H - M T E E P R K R K G S D S D -	HARNT4
1	MAA T T A N P E M T S O V P S L G P A L A S G N S G P G I Q C G G A I V O R A I K R R P C L D D D D G E C N S K F	HBMAL1b
1	MAA T T A N P E M T S O V P S L G P A L A S G N S G P G I Q C G G A I V O R A I K R R P C L D D D D G E C N S K F	HARNT
59 P S Q V E D G E H Q V K I M K A F I R E A H S Q I E K H R R D K M N N L E E L S A M I P Q C	HARNT4
47 O E S D T D K D D P H G R L E Y T E H Q G R I K N A R E A H S Q I E K H R R D K M N N L E E L S A M I P Q C	HBMAL1b
60	L R C D D D O M S N D K E R F A R S D D E Q S S A D K E R L A R E N H S E I E R R B R N K M T A Y I T E L S D M V P T C	HARNT
104	N P M A H K L D K L T V L R M A V O H L R S L K G L I N S V V G S N Y R P S F L Q D N E L R H L I L K I A E G F L F V V	HARNT4
103	N A M S R K L D K L T V L R M A V O H M R I L R G A I N P Y T E A N Y K P I F L S D D E L K H L I L R A D G E L F V V	HBMAL1b
120	S A L A R K P D K L T I L R M A V S H M K S L R G I G N T S I D G S Y K P S F I T D O E L K H L I L E A D G E L F V	HARNT
164	G E H G K I L F V S K S V S K I L N Y D Q A S L T G G S L F D F L H P K O V A K V K E O L S S F D I S P R E K L I D A	HARNT4
163	G O R G K I L F V S E S V F K I L N Y S O N D L I G O S L E D Y L H P K O I A K V K E O L S S F D I A P R E R L I D A	HBMAL1b
180	S C E T G R V V Y V S G S V I P V L N O P O S E W F G S T I Y D Q V H P D D V D K L R E O L S F S E N A L T G E I L J L	HARNT
224	K T G L Q V H S N L H A G R T R V Y S G S H R S F F C R I K S C K I S V K E E H G C - - - L P N - S K K - - - K E	HARNT4
223	K T G L P V K T D I T P G P S E L C S G A R R S E F C R M K G N R P S V K V E D K D - - - F P S T C S K K - - - K A	HBMAL1b
240	K T G - T V K E G O O S S M E M C M G S H R S F I C R M F C G S S S V D P V S V N R L S F V R N R C R N G L G S V K D	HARNT
274	H R K - E Y I T H C I G Y L R S W P P N I V G M E E E R N S K K D N S N E T C L V A I G R L O P Y I V P O N - S G E I N	HARNT4
275	D A K S F C I I H S T G Y L K S W P P T K M G L D E D N E P D N E G N L S C L V A I G R L H S H V V P O V N G E I R	HBMAL1b
299	G E P H E V V H C I G Y I K A W P P A G V S L P D D D P E A G Q G S K E - C L V A I G R L Q V T S S P N C T D M S N V	HARNT
332	V K P T E F I T R E A V N G K E V Y V D Q H A T A I L G Y L P O E L I G T S C Y E F H O D D I H N N I T O K K A V L O	HARNT4
335	V A S M E Y V S R T A I D G E V E V D Q H A T A I L A Y L P O E L I G T S C Y E F H O D D I G H L A E C H R O V L O	HBMAL1b
358	C P T E F T S R H N I E G I T E V D H R C V A T V G Y C P Q E L L G K N I V E F C H P E D O O L R O S F C O V V K	HARNT
392	S K E I I T D S V K F B A K D G S E I L K S O W - S F T N P W I K E L E Y I V S V N I L V - - - - - L G H	HARNT4
395	T R E K I T U N C M K E K I K D G S E I L K S R W S F S M N P W I K E V E Y I V S T I N I V - - - - - L A N	HBMAL1b
418	L K G O V L S V M F R E S K N Q E W L W M R T S S E T E C N P Y S D E I E Y I C T I N T N V K N S S O E P R P T L S N	HARNT
442	S - E - - - P G E A S E - - - - - L P C S S O S - - - - - S E S S R	HARNT4
445	V L E - - - G G D P T E P - - - - - O I T A S P H S M D S M - - - - - P S G E G G P K	HBMAL1b
478	T I O R P Q L G P T A N L L E M G S G O L A P R O C Q O O T E L D M V P G R D G L A S Y N H S O V V C P V T T T G P E	HARNT
483	C S C M - - - - - S V P G M - - - - -	HBMAL1b
476	H S K P L E K S D G L F A Q D R D P R F S E I Y H N I N A D O S K G I S S S T V P A T Q O L F S Q G N T F P P T P R P A	HARNT
538 S T G I V L G A G S I G T D I A N E I L D O R L Q S S S Y L - - - - -	HARNT4
472 P G G T R A G A G K I G R M A E I M E I H E I R G S S P S - - - - -	HBMAL1b
485	E N F R N S G L A P E V T I V Q P S A S A G O M L A C I S R H S N T Q G A T T W T P T R S G F S A Q Q V A T O A T	HARNT
503 D D S S P T G L M K D I H I V N C R S M S N K E L F P P S P S E M - - - G E E A T R O N	HARNT4
516 S C G S S P L N I T S I P P D A S S P G K I L N G G T P O I P S S G L S S G A C E	HBMAL1b
658	A K T R T S O F G V G S F O T P S S F S M S L P G A T A S P G A A Y P S T N R C S N F A P E T G O A G O F O T	HARNT
545	O S T V A V H S H E R L S D G A Q L D F O A L C D N - - - - - D D T A M A A F M N Y L E A E G L G D P - - - G	HARNT4
561	N P G Y P V S D S S I L C E N P H I G I D M I D N D Q G S S S P S N D E A A M A V I N S L L E A D A G L G G P - - - V	HBMAL1b
718	R T A E G V G V W P Q W Q G Q C P H H R S S S E Q H V Q O P P A Q O P G O P E V F O E M L S M L G D Q S N S Y N N E	HARNT
594	D F S D I - - - - - O W T I - - - - - (SEQ ID NO:19)	HARNT4
618	D F S D I - - - - - P M P I - - - - - (SEQ ID NO:21)	HBMAL1b
777	E F P D L T M F P P F S E - - - - - (SEQ ID NO:22)	HARNT

FIG. 3A

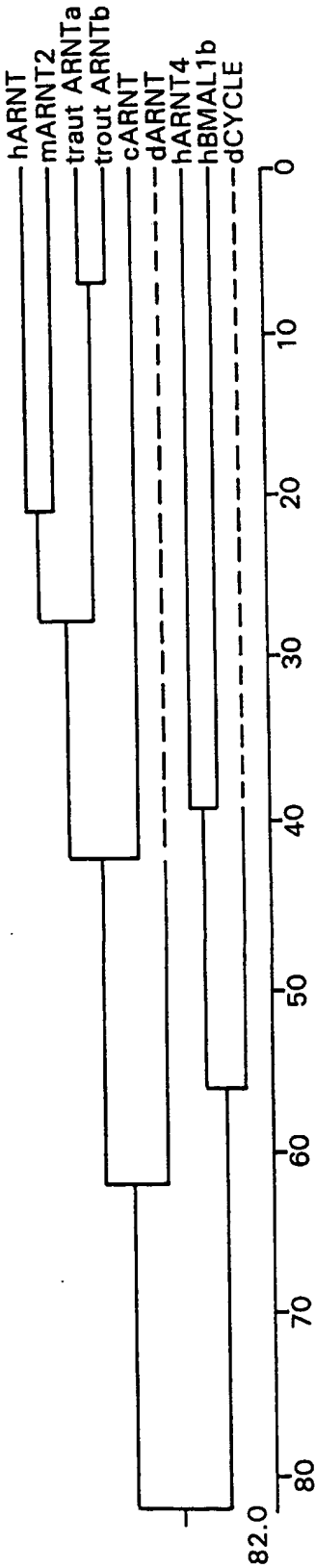
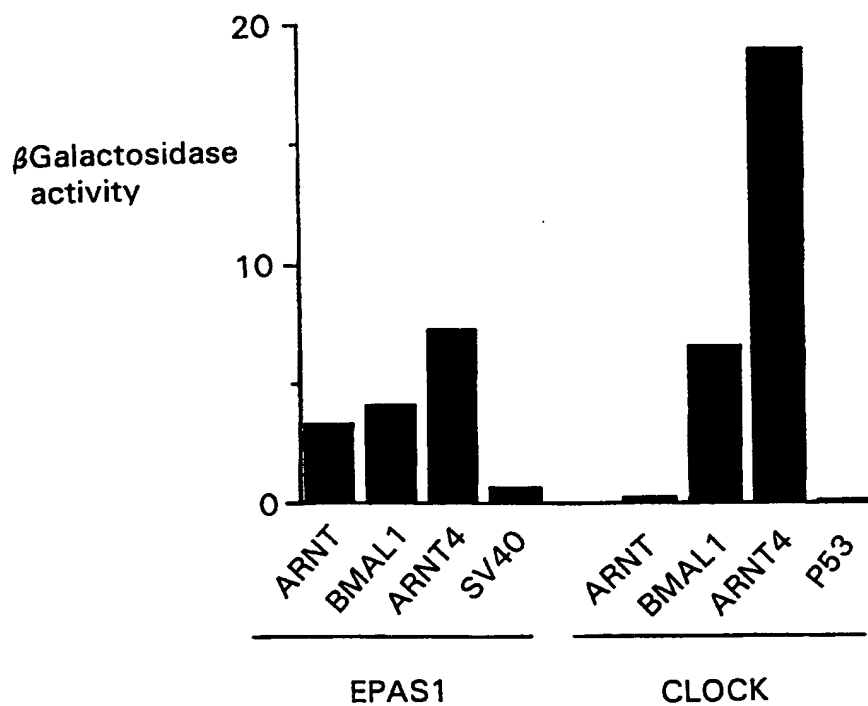
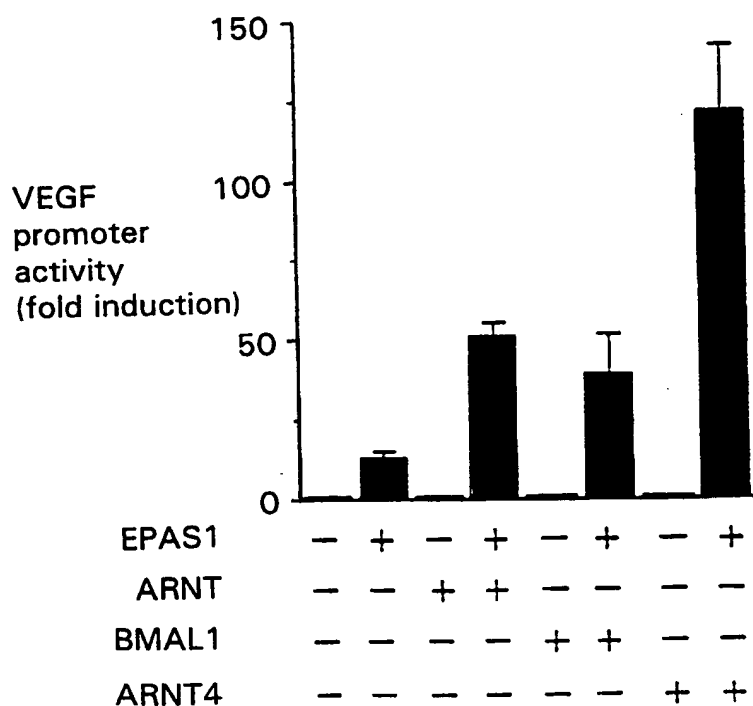


FIG. 3B

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**FIG. 4****FIG. 5**

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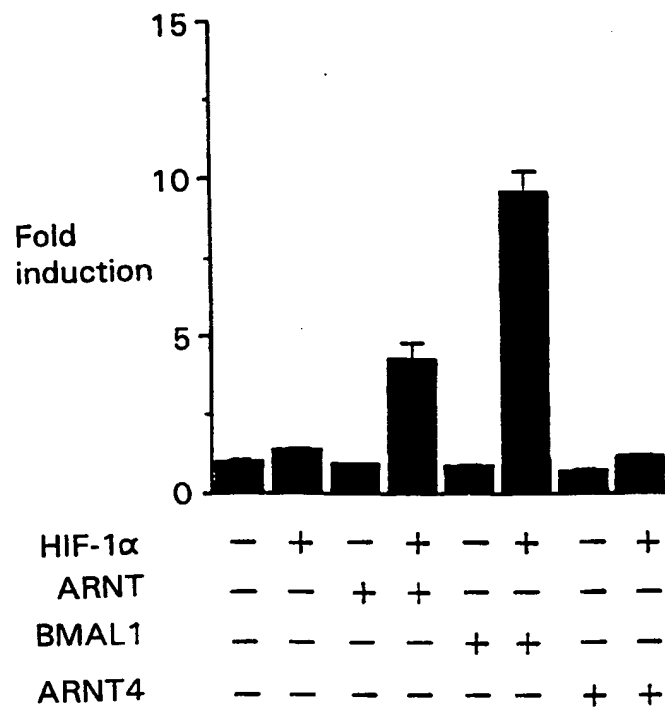


FIG. 6